Investigations into *Mycobacterium marinum* Interacting and Crossing Fish Gut Epithelia: Evidence for Inducing a Protective Gut Mucosal Immunity by a Live Vaccine Candidate

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DEDICATION

I dedicate this thesis to my family for their support and affection, especially my brothers, Paul and Vincent. Lastly, I would like to dedicate this thesis to Dr. Ennis, for allowing me to enter his lab with little experience and challenging me to become a competent graduate student. And cheers to my friends, Jack, Sutter, and Chu, among others.

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Chapter 1

Introduction

1.1. *Mycobacterium* genus

The genus *Mycobacterium* is classified as an aerobic, non-spore forming, acid-fast bacilli. Mycobacteria range in size from 0.2-0.6 μm in diameter to 1-10 μm long (Gauthier and Rhodes, 2009; Jacobs et al., 2009). All species in this genus have a very high genomic DNA GC content of between 60-70%, a feature shared with closely related genera *Nocardia* and *Cornyebacterium* (Saviola and Bishai, 2006). The *Mycobacterium* genus encompasses more than 130 species and 11 subspecies that were initially identified based on growth rate, pigmentation, and clinical significance (Stahl and Urbance, 1990). As such, they were taxonomically subdivided into two arbitrary categories: slow growers and rapid growers. The rapid growers are those species that form colonies on solid media in less than a week, while slow growers require more than a week and as long as one month for colony formation. More recently, molecular phylogenetic analyses have been used to differentiate species of this genus, which complements previous growth-rate criteria. Genes encoding for the 16S rRNA, which are widely used sequences for phylogenetic analysis due to their universal distribution and high conservation have been employed to infer relationships among species (Figure 1.1) (Rogall et al., 1990; Ninet et al., 1996). *Mycobacteria* with high sequence homology are closely related and are on neighboring branches of the phylogenetic tree (Saviola and Bishai, 2006). Depicted on the phylogeny is the division among rapid and slow growing organisms. Interestingly, most rapid growers, with the exception of *M.*

chelonae and *M. absenses*, have two copies of the 16S rRNA, while slow growers have only one copy (Tortoli, 2003). Examples of slow growing mycobacterial members include *M. tuberculosis*, *M. leprea, M. avium*, and *M. marinum*, whereas *M. smegmatis* and *M. fortuitum* are two fast-growing members. Except for the former species, fast growers are thought to be saprophytic. Another target for taxonomic identification includes the 65 kilodalton (KDa) heat shock protein gene (*hsp65*). Recent genomic studies show that *M. marinum* and *M. tuberculosis* share over 85% amino acid identity with over 3,000 orthologous genes. Furthermore, whole genome analysis of mycobacterial species suggests *M. marinum* is the closest genetic relative to the *M. tuberculosis* complex (Stinear et al., 2008), offering support for *M. marinum* being employed as a prominent surrogate pathogen to study *M. tuberculosis* (Figure 1.8). Additional shared characteristics of this genus include the structure of their cell envelopes, whose primary components are long chain fatty acids (i.e., mycolid acids). Two fatty acid chains constitute mycolic acids: a 56 to 64 carbon meromycolyl chain and a 24 to 26 carbon fatty acyl chain. A novel Fatty Acid Synthesis-2 (FAS-2) biochemical pathway produces the unique chains. Fatty acids in the common FAS-1 pathway can be shunted into the FAS-2 pathway. Enzymes involved in the FAS-2 pathway represent potential potent anti-mycobacterial drug targets (Veyron-Churlet et al., 2008). Mycolic acids are purported to assemble and form an outer envelope (Saier, 2008). The lipids in this structure account for up to 40% of the total cell weight and are responsible for their resistance to many other staining processes used for other bacteria. Though very common among members of this genus, the diverse composition of these mycolic acids can be diagnostic for species identification (Tortoli, 2003). Additionally, mycobacterial

fatty acid outer cell membranes have been accredited to be the first line of defense against potential lethal environmental conditions, although the lipid envelope carries porins, permitting passive diffusion of small molecules into a periplasmic space (Danilchanka et al., 2014). Better characterizing mycobacterial envelope porins may offer insight into more precise drug delivery. The outer membrane is partially responsible for important characteristics of *Mycobacteria*, such as resistance to chemical injury, low permeability to antibiotics or disinfectants, and resistance to dehydration (Danilchanka et al., 2014). Other factors involved in the survival of these organisms include biofilm formation and amoeba-association (Hilborn et al., 2006; Falkinham III, 2007; Hatfull et al., 2008; Peterson et al., 2013). Surviving in the protective environment of a biofilm allows for cooperation amongst bacteria and is beneficial to the group as well as most individual cells. For instance, the cells in the interior portion of these communities are thought to be shielded against exposure to many toxicants by the dense extracellular matrix as well as surface cells. Some mycobacteria and other organisms can also reside within digestive vesicles of amoebae, surviving and subsisting following phagocytosis (Hilborn et al., 2006).

Mycobacterial species are widespread in nature and include environmental species, facultative intracellular pathogens, or obligate intracellular pathogens. Water, soil, aerosols, protozoa, and biofilms may serve as reservoirs for a number of environmental mycobacterial species of this genus, including *M. chelonae*, *M. avium*, *M. fortuitum*, and *M. kansasii*. Mycobacterial pathology in humans is primarily presented in the form of pulmonary tuberculosis (TB) by members of the *M. tuberculosis* complex (MTC), which includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti*, and *M. microti*

(Perez-Martinez et al., 2008). These mycobacterial pathogens along with *M. leprea* and *M. ulcerans*, the etiological agents for leprosy and Buruli ulcer, respectively, are among the most burdening necrotic infectious agents in the world today for human populations (Gangelosi, 2004). All infectious mycobacteria from this genus other than the MTC group are often referred to as the nontuberculosis mycobacterial species (NTM). At times, certain members of NTM, such as the *M. avium-M. paratuberculosis* (MAP) complex, can cause pulmonary and/or disseminated infections in humans, especially in immune-compromised individuals (e.g., infants, elderly, HIV/AIDS patients). In fact, the emergence of the AIDS pandemic has accelerated the number of *M. avium* caused mycobacteria infection frequency to about 25% to 50% (Falkinham III, 1996). Under normal circumstances, many environmental mycobacteria are considered saprophytic and only cause minor human infections (e.g., *M. chelonae*). Recent studies in the United States suggest that humans are frequently exposed to a substantial amount of NTM species via showerheads. Researchers sampled showerheads to determine bacterial populations and found, in some cases, 100-fold increase in NTM populations compared to the municipal water of this community. Contact with NTM through aerosolization when showering could account for the increase in human NTM infections, especially lung infections (Pace, et al., 2009). Many of these environmental mycobacterium species can produce severe to minor infections in naturally susceptible hosts (Table 1.1). For instance, members of the MAP complex have been shown to cause disease in water buffalo, cattle, pigs, deer, horses, and birds (Biet et al., 2005). Moreover, aquatic NTM species, such as *M. marinum*, *M. chelonae*, and *M. fortuitum*, are common in poikilotherms (e.g., fish, frogs, and snakes). The focus of this thesis is

Mycobacterium marinum and its role as a causative agent of mycobacteriosis in teleost fish, transmission of these bacteria following ingestion and presumable transfer of mycobacteria across fish gut epithelia. Using oral route of infections, experiments were performed to investigate the possible induction of protective immunity against wild-type *M. marinum* in Japanese medaka (*Oryzias latipes*) by orally priming fish immunity with a live, attenuated strain of *M. marinum*.

1.2. *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (Mtb), the causative agent of human tuberculosis (TB), heavily burdens human populations. An estimated one third of the world's population is said to be currently infected with Mtb. The latest World Health Organization reports estimate 8.6-9 million new cases of TB and 1.3-2.1 deaths—mostly infants—in 2012 (WHO, 2013). Tuberculosis is presently the leading infectious disease by a single agent in humans; much work is left to be done to contain, eliminate, and eradicate the pathogen from human populations. These startling statistics come as a surprise to many Westerners: the prevalence of TB is not homogeneously distributed. Individuals living in certain parts of the globe such as South-East Asia or sub-Saharan Africa have a much higher TB incidence (Figure 1.2). This trend is said to be attributed to and exacerbated in part by differences in certain regions' wealth and developmental statuses. Medically underserved communities with high-density human populations are an ideal environment for Mtb to thrive. As noted during a 2008 census, the prevalence of TB per 100,000 inhabitants was 410 in low-income countries, 180 in lower middle-income countries, 73 in upper-middle income countries and 8 in high-income countries (WHO, 2013).

Approximately 90% to 95% of individuals exposed to TB develop a chronic, latent infection that does not result in disease presentation (Ganguli et al., 2005). Only 5 to 10% of infected individuals develop clinical disease within two years of primary infection (Styblo, 1980; Comstock, 1982). Approximately 80% of infected individuals die of other causes, with neither having knowledge of contracted the disease nor displaying signs of the disease. Exposure rarely leads to acute disease, since the vast majority of individuals are able to contain infection by mounting appropriate cell-mediated immune responses that involve formation of aggregates of immune cells (i.e., granulomas) (Segovia-Juarez et al., 2004; Ganguli et al., 2005; Tekippe et al., 2010). It has long been speculated (since the 1880s) that granulomatous aggregates entrap the bacteria and prevent further dissemination, although recent studies seriously challenge this interpretation. One aspect of this pathogen that makes it such a formidable opponent to the immune system is its ability to not only evade the host immune system by residing and propagating within hosts' immune cells but also, to survive and remain viable within these unique granulomatous structures. In order to survive in the hostile environment, the bacterial cells are thought to avoid killing in part by preventing phagosome-lysosome fusion during early infection. Later in infection, the pathogen able to survive harsh anoxic conditions present in granulomas, and are thought to lie dormant or latent for years. Mycobacteria have developed sophisticated techniques to evade effective immune responses or endure lesser immune responses. However, in infected individuals, there is a 5% to 10% chance of the disease progressing to a highly contagious form known as active tuberculosis (Bouley et al., 2001; El-Etr et al., 2004; MacGurn and Cox, 2007). Therefore, although most individuals maintain these latent

bacteria in infected tissues for a lifetime, reactivation is possible with a compromised immune system due to old age, starvation, or infectious burdens with other infectious diseases (e.g., HIV and malaria) (Bouley et al., 2001). With such a wide range of apparent survival tactics, understanding the mechanisms this pathogen employs to evade the host immune response is an essential step in the development of a successful treatment or preventative methods.

1.3. Granuloma formation

Granuloma formation results from an inflammation response due to a persistent stimuli caused by an "indestructible" or "indigestible" antigenic material, including intracellular bacteria, as a result of many conditions such as sarcoidosis, Crohn's disease, Churg-Strauss syndrome, and TB. Granuloma formation is the product of both the acquired and innate immune response that involves interaction of multiple elements, which include immune cells (T cells, dendritic cells, and macrophages), immune effectors (chemokines and cytokines), and the specific pathogen involved (Segovia-Juarez, 2004). For tuberculosis, the inflammation reaction commences when mycobacterial cells reach lung alveoli and encounter resident macrophages. These macrophages' specific function is to ingest and destroy foreign antigens. However, in the case of *M. tuberculosis* and other mycobacterial species (e.g., *M. avium* and *M. marinum*), some mycobacteria have evolved mechanisms to evade killing inside resting macrophages and use these immune system cells as their replicative niche. Hence, once inside the vacuole of a resting macrophage, the bacterial cells replicate. In contrast, mycobacteria that encounter activated macrophages are usually ingested and killed. After the innate immune cells' initial response to eradicate this pathogen, chemokines

and other signals are produced to activate the adaptive immune response, leading to a cascade of events to halt the spread of the disease. The immune cells responsible for the start of these events include macrophages, which secrete chemokines and cytokines (e.g., interleukin-1b (IL-1b), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6)) to attract dendritic cells (antigen presenting immune system cells) and neutrophils to the primary site of infection. Once recruited, these phagocytes engulf extracellular bacilli and migrate to local lymph nodes and present antigens to naïve lymphocytes (e.g., helper T cells, cytotoxic T cells). Activated lymphocytes (e.g., T helper cells-1) produce cytokines such as interferon gamma (IFN-γ), interlukin-2 (IL-2), and interlukin-12 (IL-12), substances that activate macrophages to produce reactive nitrogen intermediates and are also responsible for the lysis of infected macrophages (Flynn and Chan, 2001). These activated macrophages and neutrophils are critical components in the phagocytosis and killing of extracellular bacilli. Continual recruitment of lymphocytes, macrophages, and other immune cells to the site of infection results in formation of complex, tightly aggregated granulomatous spherical structures (Segovia-Juarez et al., 2004). Since the late 1800s, these structures were projected to be a tactic by which the adaptive immune response uses to achieve and maintain latency in tuberculosis (Segovia-Juarez et al., 2004; Andersen, 1997; Saunders and Cooper, 2000). However, recent studies suggest this classical model for granuloma formation may be far more complex. Studies in transparent zebrafish hatchlings and Japanese medaka infected with *M. marinum* show that granuloma formation occurs rapidly; thusly, granuloma formation is thought to occur before an adaptive immune response (Broussard and Ennis, 2007; Davis and Ramakrishnan, 2009). Recent studies

show granuloma formation coincides with an acceleration in bacterial population expansion (Volkman et al., 2004). Moreover, mycobacteria were observed to be actively transported in macrophages between granulomas, suggesting the bacterial cells are not contained inside granulomas. Current thought is that the major role of granulomas is to protect mycobacteria from host immune responses (Davis and Ramakrishnan, 2009). Figure 1.3 shows multiple examples of granulomas.

1.4. Adaptive immune response

Adaptive immune responses represent the key factor for developing efficient and impactful vaccines. The adaptive immune system allows the immune system to store information in the form of memory B cells. In addition, adaptive immunity enables the immune system to specifically recognize a foreign antigen and immediately activate biochemical pathways that culminate in the production of specific antibodies.

The major type of adaptive immune response is the mucosa-associated lymphoid tissue (MALT). Bacteria typically colonize surfaces after invading mucosal surfaces. The MALT culminates with the production of specific antibodies (IgA) that are produced and secreted across the epithelia and into the mucosal layer. In mammals, antigen presenting cells (APC) sample bacteria present in the gut lumen. The bacteria are phagocytized by macrophages and bacterial antigens are presented to T-helper cells. In turn, T-helper cells will stimulate memory B cells to process the antigen and produce IgAs. These IgAs bind to appropriate antigens and prevent the bacterium from binding to host cell surfaces. The bulk of the macrophages, B cells, and T cells associated with APC reside specifically in the lamina propria of mucosal surfaces (Wilson et al., 2011).

Some pathogens have developed techniques to exploit the APC system and survive the macrophage's challenge and persist in host cells.

Vaccines manipulate the adaptive immune response to mount appropriate protection against a subsequent virulent challenge. More specifically, oral vaccines stimulate the gut-associated lymphoid tissue (GALT) system to induce protection against a virulent challenge along the mucosal linings of the gut. The GALT is a subdivision of the MALT. As such, T cells and B cells differentiated to protect against a specific antigen can migrate to other mucosal linings in the host body and confer protection there. However, the protection is confined to the MALT and does not protect against a non-MALT induced challenge by the pathogen (Wilson et al., 2011). Being that *M. marinum* infects medaka orally, inducing a GALT response to *M. marinum* may confer protection against a virulent *M marinum* challenge.

Adaptive immunity is said to have first evolved with the appearance of jawless fish around 550 million years ago. Though, immunoglobins (Ig) and T cell antigen receptors (i.e., CD4+, CD8+) are thought to be lacking in jawless vertebrates. Additionally, the specialization of immunoglobins for systemic and mucosal immunity is thought to have evolved along with tetrapod evolution (Flajnik, 2010). Recent discoveries challenge this paradigm.

Studies by Zhang et al. (2010) characterized a novel immunoglobin (IgT, teleost) in rainbow trout. The protein level analysis shows the immunoglobin to be monomeric in serum but polymeric in the gut mucous. IgT is thought to be functionally analogous to mammalian secreted IgA (sIgA). The researchers also present evidence that a distinct B cell lineage expresses only IgT on the cells' surface; the IgT+ B cell lineage represents

the main B cell subset in the GALT of rainbow trout. To further study the immunoglobin, researchers exposed trout to a pathogen to characterize IgT resposnses. IgT expression was predominately confined to the gut mucousal layer. Moreover, the majority of the microbial cells in the gut where coated with IgT. Collectively, the studies imply that mucosal and systemic immunity specialization evolved in teleost, challenging the view that this immune system specialization first occurred in tetrapods. IgT and the mammal and bird equivalent, sIgA, are functionally similar but phylogenetically distant or non-related, suggesting their evolution into mucosal immunoglobins most likely occurred independently via convergent evolution (Zhang et al., 2010).

Vaccination experiments were created to gain insights into potential players in adaptive immunity in medaka. *In silico* analyses of the medaka genome by Irene Salinas indicate the absence of an IgT equivalent. However, CD8 cytotoxic T cells, IgM, secreted IgM, Interleukin-12, Interleuken-2, and Interferon-gamma have all been implicated as potential players in mobilizing medaka's adaptive immunity.

1.5. Vaccination against mycobacteriosis

Mycobacterial mutants have been constructed to study genes responsible for virulence. One of the best characterized gene sets reside within the region of difference-1, called "RD1" (Figure 1.4). This is a 9.5 kilobase interval present in a number of mycobacterial pathogens, but was initially defined by a deletion present in the *Mycobacterium bovis* vaccine strain and all derivatives collectively called Bacili Calmette-Guerin (BCG). BCG has long been used as the major anti-tuberculosis vaccine (Smith et al., 2008). The RD1 region is also absent in the genomes of other highly

attenuated strains of *Mycobacterium*, including *M. marinum* and *M. tuberculosis* (Volkman et al., 2004). The RD1 locus encodes for a type VII secretion system (ESX-1) responsible for the exportation of proteins necessary for host interaction and pathogenesis. This secretion system has been linked with multiple pathogenic characteristics related to both *M. tuberculosis* and/or *M. marinum* including: phagosome maturation arrest, granuloma formation, cytolysis, and necrosis (MacGurn and Cox, 2007; de Jonge et al., 2007). Two known virulent factors secreted from this system include the "early secreted antigenic target-6" (ESAT-6) and "culture filtrate protein 10" (CFP-10), whose genes are also situated within the RD1 interval, leading to phagosome trafficking modulation (MacGurn and Cox, 2007). Mutants of ESX-1 secretion pathway exhibit multiple defects, such as inability to lyse host cells and reduced intracellular survival. ESAT-6 mutants were also shown to be deficient in forming mature granulomas and fail to recruit other bacteria within the same structure (Davis and Ramakrishnan, 2009).

As of now, BCG is the only accepted anti-TB vaccine that has been widely applied. This vaccine is somewhat protective against acute types of TB (i.e., miliary TB, disseminative TB, TB meningitis) especially in infants. Figure 1.5 depicts the global distribution of BCG administration to human populations across the globe. The U.S.A. does not administer BCG to help track any potential domestic outbreak of TB by testing for seroconversion. Studies indicate that BCG provides little if any protective qualities against tuberculosis in adolescents and adults (Cosma et al., 2004). Additionally, BCG is not recommended for use in HIV-positive infants, due to the risk of the live BCG spreading to others. With an increase in cases of drug resistant tuberculosis, avoiding

infection incidences via efficient vaccination programs would presumably decrease cases of drug resistant tuberculosis (WHO, 2013). An attenuated, live *M. marinum* strain would not only confer protective immunity in fish but may be cross-protective to other mycobacteria and offer insights into improving anti-TB vaccines for human populations.

Current and recently completed anti-TB vaccine clinical trails will offer important leads into novel ways to provide more efficient protection against TB via vaccination. The recently completed MVA85A Phase II-b vaccine trail was the first anti-TB vaccine study done in a high-incidence TB population. Though the vaccine did not prove to be more effective than BCG, the study paved the way for expanded anti-TB vaccination studies in the future (Tameris et al., 2013).

There are two guiding theories for improvements to anti-BCG vaccines: firstly, researchers are searching for a more efficient agent to replace BCG or, secondly, identifying a supplemental booster-dosage to complement BCG administration (Evans et al., 2013). In the latter "prime-boost" strategy, neonates would continue to receive BCG vaccines, these subsequent boosters are applied to increase the efficacy and extend the duration of protection. Since it is well documented that individuals who received BCG during their infancy, lose protection upon reaching adulthood, stimulating the immune system with a booster later in life may confer additional protection against TB in adults. Computational research models using Southeast Asia as a demographic model express that an anti-TB vaccine with 60% efficacy in infants could lead to a significant decline in TB burden in the region (Abu-Raddad et al., 2009). Additional applications of the model indicate that if an anti-TB vaccine for adults with similar efficacy were

administered through massive public health efforts, the TB burden would further decline (Abu-Raddad et al., 2009). Though strategies to implement such grand-scale anti-TB projects remain illusive, improvements to the vaccine would significantly decrease global TB burden in humans. There are currently 12 vaccine candidates in various stages of clinical trials, which may offer hope for billions of people (WHO, 2013).

Adaptive immune responses represent a key factor to developing impactful vaccines. The adaptive immune system elicits long-term, specific immunity against a pathogen. Live vaccines must meet a handful of requirements to be considered as potential candidates. Firstly, vaccines must be attenuated enough to be fully cleared by the host. However, a vaccine cannot be so highly attenuated to the point that the bacteria fail to illicit long-term, adaptive immune responses. If too highly attenuated, the bacteria are cleared too quickly and fail to illicit a full adaptive immune system response. Thus, there is no protection against a subsequent challenge. Effective live vaccines elicit a full and robust immune response, manifested by the differentiation of B plasma cells (memory B cell) capable of immediately producing specific antibodies upon activation and clearance of the infectious agent by the host immune system.

Adjuvants and boosters help supplement vaccines. Frequently, adjuvants and boosters are administered in smaller doses compared to vaccinations, usually given years after the initial vaccination. Additionally, an adjuvant may be a viral construct to express just one bacterial gene product and may be wholly attenuated. The one gene in the virus could be a potent immune system stimulant and increases protection against subsequent challenges by a given agent. Since BCG-vaccinated children lose protection

against TB when approaching adulthood, adjuvants and boosters in humans have been implicated as potential tools to help improve the efficacy of BCG in adults (WHO, 2013).

1.6. *Mycobacterium marinum* and its use as a TB model

1.6.1 Mycobacterium marinum

Mycobacterium marinum (Figure 1.6) is a known fish pathogen. Like *M. tuberculosis*, *M. marinum* is one of the closest relatives to the TB complex. Like *M. tuberculosis*, *M. marinum* is also an intracellular pathogen able to survive and replicate within macrophages. Fish mycobacteriosis was first described in codfish by Alexander (1913), and *M. marinum* was isolated by Aronson (1926) from a coral fish in the Philadelphia aquarium in 1926. *M. marinum* is known to be ubiquitous in many bodies of fresh, brackish, and salt water environments, causing infection in at least 60 species of fish (Chinabut, 1999) and some specie of amphibians (El-Etr et al., 2001). This bacterium has been shown to replicate within aquatic protozoan organisms, such as *Dictyostelium polyphaga* (Solomon et al., 2003; Hagedorn and Soldati, 2007; Peterson et al., 2013). Systemic infection by *M. marinum* can produce severe disseminated disease (i.e., mycobacteriosis) in colonies of striped bass, one of the most economically valued aquaculture fish species in the USA (Osland et al., 2008). Indeed, previous estimates indicate that *M. marinum* outbreaks in such aquaculture facilities in the United States alone have caused more than 125 million dollars in economic loss annually (Cirillo, 1999). Also at risk due to increased mycobacteriosis incidence are research facilities housing aquatic species such as medaka, zebrafish, and *Xenopus laevis* (Broussard and Ennis, 2007; Lawrence et al., 2012). Hence, studies elucidating methods of transmission and insights into possible vaccines offer more insights for the containment but also

protection from mycobacterial diseases in research and aquaculture facilities (Jacobs et al., 2009).

As with *M. tuberculosis*, this fish pathogen is an aerobic acid-fast bacillus that ranges in size from about 0.2-0.6 μm wide and 1-6 μm long (Chinabut, 1999). *Mycobacterium marinum* (Biosafety level 2) is easily manipulated with reduced risk to researchers, compared to *M. tuberculosis* (Biosafety level 3) with convenient culturing on Middlebrook liquid and/or agar media at 28° to 30° C. *M. marinum* has a four hour generation time with colony formation at seven to eight days, which is some three- to four-fold less time than *M. tuberculosis*. Based on the time required for visible colony formation, this bacterium is classified as one of the "slow growing" *Mycobacterium* species (Shinnick and Good, 1994), which are typically the human and other animals' pathogens. Initially, *M. marinum* colony morphology is white, round, rough, and opaque. However, following exposure to light, colonies turn yellow in color as a result of production of yellow carotenoid pigments. This light-induced yellow pigmentation of colonies is employed as a unique diagnostic characteristic for *M. marinum.* Other diagnostic microbiological properties include urease (+), thiopen-2-carboxylic acid hydrazide sensitivity (+), arylsulfatase (+), pyrazinamidase (+), tween hydrolysis (+), catalase (-), and nitrate reduction (-). Molecular restriction enzyme assays have also been developed to differentiate *M. marinum* from other common NTMs (Talaat et al., 1998). In laboratory settings, this bacterium can easily be manipulated to engineer multiple mutants for characterization of genes important in a number of processes including: bacterial pathogenesis in a live host or specific steps in interacting with

macrophages (Barker et al., 1997; Gao et al., 2003; Ramakrishnan, 2004). *M. marinum* colony morphology can be seen in Figure 1.6.

1.6.2 *Mycobacterium marinum* as a model organism for *Mycobacterium tuberculosis*

Due to the highly contagious nature and slow growth of *M. tuberculosis*, alternative methods, or surrogate pathogens, have been employed to study mycobacterial pathogenesis in order to reduce the danger of exposure to researchers, and increased growth rate increases the pace of research. Since *M. marinum* is closely related to the MTC, shares many of the same virulence genes, and presents similar pathology in fish, this pathogen has been considered a potential surrogate pathogen. These studies require the development of tractable animal models that are naturally infected by the relevant microorganisms that mimic aspects of infection seen with tuberculosis in humans. As noted above, aquatic animal hosts, such as zebrafish, goldfish, and frogs have been employed in experimental infections with *M. marinum* (Talaat et al., 1998; Davis et al., 2002; Prouty et al., 2003). A chronic-infection model was developed by Broussard and Ennis (2007) using *M. marinum* and the small laboratory fish, Japanese medaka (*Oryzias latipes)* as the model host. The *M. marinum*-medaka infection pair was found to produce life-long TB-like chronic infections with similar pathology, including granulomas. Clinically reported human infection by *M. marinum* is fairly unusual, but can occur in the cooler extremities, like the digits. Infections in these sites are likely due to inoculation of cuts of the skin and the lower temperature requirements (25° to 30° C) for the microbes. Factors that contribute to the success of this infection model include: an authentic, natural host-pathogen interaction and the pathogen's ability to infect the host producing similar pathology

that exists in human tuberculosis. For instance, invasion of host macrophages and elicitation of an inflammatory response leading to formation of granulomatous lesions, the hallmark structure of human tuberculosis, is also seen in teleost fish or other poikilothermic species infected with *M. marinum*. Mice experimentally infected with *M. marinum* held below 30° C developed pulmonary lesions while those kept at higher temps (34° C) did not (Petrini, 2006). Also, certain *M. marinum* strains can be adapted to grow at 37° C and cause systemic infections in mice (Ramakrishnan et al., 2000). Hence, temperature is postulated as one determining factor of the site of infection and not the host. Interestingly, humans infected via cut or abrasion by *M. tuberculosis* can also have dermal localization without systemic spread and that is pathologically indistinguishable from *M. marinum* caused dermal infection (Ramakrishnan, 2004).

Recently, there has been an increase in the use of *M. marinum* as a surrogate model organism to study mycobacterial pathogenesis in hosts like zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), medaka (*Oryzias latipes*), and leopard frogs (*Rana pipiens*). A study by Gauthier et al. (2003) observed granuloma disintegration and dissemination of *M. marinum* in striped bass 26 to 32 weeks post injection, similar to reactivation of latent TB due to failure of the host immune system. Leopard frogs chronically infected for approximately a year have been chemically stimulated to develop lethal *M. marinum* disease after administration of corticosteroid that supresses the immune system (Bouley et al., 2001). This suggests that *M. marinum* infection in poikilotherms may be useful in modeling primary progressive or secondary reactive tuberculosis as seen with human patients (Gauthier and Rhodes, 2009). When compared using commonly employed molecular genetic markers (e.g., 16S rRNA) to *M.*

tuberculosis complex, *M. marinum* was found to be one of its closest relatives. More recently, *M. marinum*'s genome was compiled and annotated. The genome size is 6.6 megabases (Mb) and is approximately 1.5 times that of *M. tuberculosis* (4.4 Mb). Speculation suggests that the extra *M. marinum* genome encodes determinants for environmental survival while *M. tuberculiosis* may have undergone reductive evolution from an *M. marinum*-like parent, becoming a more specialized intracellular pathogen (Ramakrishnan et al., 2004; Stinear et al., 2008). Nevertheless, *M. marinum* shares at least 85% amino acid identity to over 3,000 orthologous genes of *M. tuberculosis* (Stinear et al., 2008) and a 99.4% identity with the 16S rRNA gene (Figure 1.1). With such extensive genetic similarities and commonality in pathology, *M. marinum* has proven to be an excellent model for understanding the pathogenesis of tuberculosis. Furthermore, virulence determinants are shared between the two species; for instance, orthologous *M. marinum* virulence genes can complement defects in *M. tuberculosis* genes (Cangelosi et al., 2004).

1.6.3. Utilizing Japanese medaka (*Oryzias latipes*) to study *M. marinum*

Because of the recent increase in the use of small-bodied fish species as model organisms (e.g., zebrafish, goldfish, and medaka) at various research facilities, mycobacteriosis outbreaks have become a major concern in such facilities (Sanders and Swains, 2001; Kent et al., 2004; Jacobs et al., 2009; Lawrence et al., 2012). Japanese medaka (*Oryzias latipes*) is a small freshwater fish specie that becomes sexually mature in about three months with a life span that may extend to three years (Sanders and Swains, 2001). They are indigenous to some regions of Asia, particularly small bodies of water in Japan, where they can be readily observed in rice paddies. Because of their

small size (150-300 mg), length (3.5 cm), and broad temperature tolerance (4° to 40° C), medaka are easy to maintain and propagate under laboratory conditions. Under optimum conditions, a healthy female can lay up to 30 eggs in a day. Medaka have been routinely used for toxicology, embryology, and molecular mutagenesis studies for nearly 100 years. Recently, medaka fish are emerging as a model to study human tuberculosis (Broussard, 2007; Broussard and Ennis, 2007, Broussard et al., 2009). With these experimental infections and other reported cases of *M. marinum* outbreaks in medaka colonies (unpublished data from A. Camus, University of Georgia and W. Hawkins, University of Southern Mississippi), we believe that medaka colonies are commonly infected with *M. marinum*.

1.7. Mycobacteriosis

1.7.1 Mycobacteriosis outbreaks in aquatic animals

Mycobacteriosis is one of the most common infectious diseases of many captive, wild, and research fish species (Harriff et al., 2007). Environmentally, the distribution is worldwide, infecting both cultured and natural populations of freshwater, estuarine, and saltwater fish (Santos, 2002). To date, *M. marinum* infections have been reported in over 160 different fish species across the globe (Chinabut et al., 1990; Kaattari et al., 2006), including a few of the most economically and recreationally important species such as striped bass (*Morone saxatilis*), sea bass (*Dicentrarchus labrax*), and tilapia (*Oreochromis mossambicus*). A study by Giavenni et al. (1980) observed infections in numerous economically relevant tropical fish species as well as some handlers involved in day-to-day animal husbandry. Recently, during the ongoing Chesapeake Bay epizootic crisis, over 60% of striped bass sampled in the Maryland portion of the bay

were found to be infected with mycobacteria. This crisis has lasted for more than a decade (1997-present) and has caused approximately 200 million dollars in annual economic loss thus far. Other fish species in the bay that have tested positive are blueback herring (*Alosa aestivalis*), winter flounder (*Pleuronectes americanus*), striped killifish (*Fundulus majalis*), largemouth bass (*Micropterus salmoides*), and weakfish (*Cynoscion regalis*) (Ottinger and Jacobs, 2006). An outbreak in Portugal (dos Santos et al., 2002) was reported to infect a turbot (*Psetta* maxima) farm where 80% of seemingly healthy fish were found to be infected. In the red sea, the first identified mycobacteriosis cases were in the early 1900s in a mari-cultured (i.e., seacaged) sea bass population (Ranger et al., 2006). Since then, there seems to be an increase in infection levels in wild fish of the surrounding area as a consequence of interaction between farm fish stocks and local fish populations (Diamant et al., 2000). Other systems suffering from constant cases of mycobacteriosis are recirculating aquaculture systems such as those found in aquaculture, wholesale, and retail facilities. Mycobacterosis outbreak in cultured fish was first documented in the late 1950s when mortality among hatchery-raised *Oncorhynchus* and *Salmon* spp. dramatically increased with 100% morbidity in some populations (Wood and Ordal, 1958). Since then, mycobacteriosis outbreaks have become common occurrence in the fish food industry. As one of the fastest growing food producing sectors (Cole et al., 2009), which supplies approximately 40% of the world's fish food, the health of fish in the aquaculture industry is of utmost importance. According to Francis-Floyd (1999), during an outbreak in such an environment, infection rates can vary from 10% to as high as 100% (Cole et al., 2009). Of late, the rapidly growing sector of hybrid striped bass (*Morone*

chrysops x *Morone saxatilis*) aquaculture, producing more than 30 million dollars in fish annually is highly susceptible, especially the juveniles, to mycobacteriosis (Osland et al., 2008). Thus far, there is no known effective method of curing such outbreaks but to cull and dispose infected populations, causing extreme profit losses. Hence, preventing such an occurrence should be a top priority. Consequently, factors that promote the establishment of mycobacteriosis within a given aquaculture system need to be identified to decrease chances of exposure and prevention or reduce the spread of the disease throughout the whole colony or entire populations in the wild.

1.7.2 Mycobacteriosis pathology and causative agents in aquatic animals

Environmental *Mycobacterium* species have long been recognized as the causative agent of mycobacteriosis. *M. marinum*, *M. fortuitum*, and *M. chelonae* are the most commonly isolated bacterial species in fish, with *M. marinum* being the most common and causing the greatest impact (Rhodes et al., 2001). A number of these environmental species are also capable of causing typically mild human infections following inoculation of cuts or scratches (Giavanni, 1980). Recently, several newly emerging *M. marinum* isolates have been identified in the Red and Mediterranean Seas that are associated with severe pathology (Ranger et al., 2006). Their increase in pathogenesis was postulated to be linked to the presence of a secreted toxin (i.e., mycolactone), originally found in *M. ulcerans*, the third most common human mycobacterial pathogen (Johnson et al., 2005). However, more recent studies in medaka indicate that a mycolactone-bearing *M. marinum* was not significantly more virulent in medaka than the standard *M. marium* type strain used in our laboratory infections (Mutoji et al., 2011; Mosi et al., 2012). Neither *M. tuberculosis* nor *M. leprae* have been

isolated from water and have very narrow host ranges, unlike their environmental counterparts (e.g., *M. marinum*). For instance *M. tuberculosis* is an obligate intracellular pathogen of humans with no significant environmental reservoir, while some environmental mycobacterial species are ubiquitous in the environment and are readily isolated in soil, water sources, and environmental protists (e.g., amoebae) (table 1.1). Some environmental mycobacteria are able to survive for months or even years in extreme environments, one of which is temperatures below 20° C (Beran, 2006). A survey by Collins et al. (1984) isolated mycobacteria from 67% of all water samples collected from natural, treated, and animal-contact sources. The similarity in pathology of fish mycobacteriosis to human TB suggests similar infection mechanisms between *M. marinum* and *M. tuberculosis*. Macrophages, one of the first antimicrobial cells recruited to mycobacterial invasion, play a very important role in the proliferation and dissemination of these pathogens from the primary area of infection to deeper target tissues to establish secondary infections. Such disseminative infections can result in chronic disease with granulomatous lesions in multiple tissues or organs (e.g., liver, spleen, and kidney), very similar to those seen in chronic human TB. In fact, the similarities in histopathology are so striking that *M. marinum* is often referred to as "fish tuberculosis" (Kent et al., 2004). Fish necrotic visceral granulomas constitute of a central area of necrosis (i.e., caseous material) surrounded by macrophages, epithelial cells and fibrous connective tissue (El-Etr et al., 2001; Jacobs et al., 2009). This infection is predominantly chronic in some fish, like goldfish and medaka, and may or may not produce external clinical signs (Talaat et al., 1998; Broussard and Ennis, 2007).

Mycobacteriosis infection and its clinical signs have been extensively studied using different fish species infected naturally and experimentally. The first description of fish mycobacteriosis was by Bataillon et al. (1897) in carp (*Cyprinum carpio*), which was reportedly infected with sputum of an active TB patient. After which, natural infection was reported in cod (*Gadus callaria*), halibut (*Hippoglossus hippoglossus*), sergeant major (*Abedufdur mauritii*), Mexican platyfish (*Platypoecilus maculatus*), and many more (Alexander, 1913; Aronson, 1926; Baker et al., 1942; Sutherland, 2002). A few of the experimentally well-studied fish species include zebrafish, goldfish, and medaka. For these studies, investigators have learned the severity of fish disease acuteness varies depending on the specie of *Mycobacterium* and the host involved. For instance, a study by Broussard and Ennis (2007) observed that *M. marinum* infected zebrafish develop a severe, acute infection, whereas chronic, life-long infections were typically observed in medaka. During acute infection, zebrafish developed visible external ulcerative lesions along with internal granulomas and often succumbed to mycobacteriosis, while chronically infected medaka displayed no overt clinical signs of the disease. Wolf and Smith (1999) also observed a more severe case of mycobacteriosis and increase mortality in striped bass compared to hybrid tilapia species experimentally infected with the same dose of *M. marinum*. As with zebrafish, striped bass exhibited external clinical signs of skin discoloration and severe ulceration. Gauthier et al. (2003) compared granuloma formation in the same hosts (i.e., striped bass) experimentally infected with the comparable doses of *M. marinum, M. shottsii*, or *M. gordnae*. They observed that while *M. marinum* caused severe internal granulomatous reaction, the latter two species at no time elicited an inflammatory

response. These studies independently, yet collectively, demonstrated that differences in the hosts and the interactions with the pathogen are the determining factor for the severity of the disease. Other previously observed clinical signs of mycobacteriosis in fish include scale loss, hyperpigmentation, and hemorrhagic or ulcerative skin lesions (Landsdell et al., 1993). A study by Wood et al. (1958) involving salmon also observed stunted appearance, brighter color, and/or lack of secondary sexual development. Abnormal gross behavioral cues such as slow swimming, lack of appetite, loss of body mass, spinal defect, and lethargy have also been observed (Gomez et al., 1993; Santos et al., 2002).

With such variation in disease manifestation, or lack thereof, investigators have initiated research to study probable routes of infections of environmental pathogens in fish as well as humans. For instance, certain environmental mycobacterial species, such as *M. marinum*, MAP complex, and *M. ulcerans*, are known human pathogens with different pathogenesis manifestation. While *M. ulcerans* and *M. marinum* cause superficial infection of the skin and/or adipose tissues, MAP are known to cause pulmonary tuberculosis infection similar to *M. tuberculosis* as well as other afflictions specific to the route of infection (e.g., gastrointestinal, intra-abdominal, etc). Therefore, environmental species that are known human and fish pathogens may share similar portals of entry in respective host species' infection routes.

1.7.3 Transmission models

Because infectious mycobacteriosis impacts many species of fish, determining the method of transmission between animals and what factors can contribute to fish susceptibility are the first steps in developing preventative methods. Speculation as to

the method of transmission have led to proposals of several plausible transmission models that could explain how fish and other poikilothermic organisms become infected by *M. marinum*. They include: ingestion of dead, infected fish or infected feed, horizontal transmission or infected animals shedding bacteria (e.g., feces) into a common environment, such as a fish tank or pond (i.e. cohabitation), vertical mechanism of transmission from mother to progeny (Jacobs et al., 2009). An additional plausible mode of transmission is inoculation through wounds or abrasions with organisms present in the water column. Thus far, most of these postulated infection models are derived from retrospective veterinary studies of infected populations, but, until recently, were not experimentally tested. With so little information available in the literature, these models need extensive testing to obtain a better understanding of the means by which a few mycobacteriosis cases can usually progress into outbreaks in wild aquatic or aquaculture environments. Other factors that could influence susceptibility include the age of the animal (e.g., hatchlings vs. adults), poor general health status and environmental stressors can all contribute to an increase in susceptibility.

1.7.4. Transmission in fish by ingestion

Transmission by ingestion has been implicated as one of the primary infection routes in fish (Bruno, 1998). This method of transmission first became apparent when feeding fish with unpasteurized fish tissues led to multiple epizootics in hatchery-reared salmon; afterwards, this practice was stopped (Belas, 1995; Jacobs et al., 2009). Later, it was also suggested that wild aquatic species can become infected during ingestion of deceased infected fish. Consistent with this view, Harriff et al. (2007)

conducted one of the only studies geared toward a controlled laboratory experiment to show possible transmission through the oral route with zebrafish-*M. marinum* model. Researchers experimentally infected fish by oral intubation and observed dissemination of the disease in some animals and mycobacterial pathology in organs such as the liver and kidney. In the same study, like Cirillo et al. (1997), observed *in vitro*, bacteria passed through amoeba, slightly increased its ability to grow within the host (i.e., zebrafish). However, efforts to duplicate these studies with oral intubation using medaka did not yield efficient infection (Broussard, 2007; Mutoji, 2011). Infection by amoebae or paramecia could be one of the natural reservoirs of environmental mycobacterium and serve as intermediate hosts, facilitating transmission of these pathogens to fish, especially for animals that consume these protists (Cirillo et al., 1997; Akleh et al., 2014). Recently, paramecia were reported as carriers of *M. marinum* or *M. chelonae* resulting in acute infections in zebrafish (Peterson et al., 2013). This discovery brought together the idea that phagocytic organisms, like environmental protozoa, including amoebae and paramecia may serve as host to intracellular pathogens prior to their evolution and invasion of complex eukaryotic organisms (Ramakrishnan, 2004). Mycobacteria may have evolved mechanisms to counteract ingestion by these unicellular phagocytes, which may have resulted in the ability to also survive phagocytosis by lymphocytes in higher-level hosts. This transition to higher species could be explained by the genetic differences that exist between environmental mycobacterial species and their facultative intracellular pathogen counterparts, which allows for both an environmental and intracellular dichotomous lifestyle. Species known to survive and replicate with *Acanthamoeba* include *M. avium*, *M. marinum*, and

M. fortuitum (Solomon et al., 2003; Adekambi et al., 2006; Jacobs et al., 2009). Another attempt was made to study transmission through the oral route in a controlled setting was by Ross (1970) in which guppies were infected with *M. marinum* by ingestion of infected salmon tissue. More recently, Mutoji (2011) showed that consumption of *M. marinum*–infected fish tissue was highly infectious for medaka. As such, additional controlled experiments are needed to validate this model as the primary route of infection.

One environmental mycobacterium known to cause infection by the intestinal route in people, especially in AIDS patients or immune-compromised individuals, is MAP. Interestingly, a member of the *M. tuberculosis* complex, *M. bovis* also causes human infection via an oral route (Saviola and Bishai, 2006). It has been determined that prior to routine pasteurization, milk served as the most common reservoir for human infection by *M. bovis*. In fact, this bacterium was responsible for at least 25% of all diagnosed human TB cases in the 1940s-1960s (Palmer, 2008). Recently, there has been an increase in MAP cases in human populations. This is primarily due to increase in prevalence of this pathogen in diary herds and the run-off, which contaminates and persists in water systems, meat, and dairy products, and poorly pasteurized milk (Taylor, 2009). In immune-competent individuals, ingestion of this pathogen results in gastrointestinal (GI) inflammation. However, in AIDS patients it may lead to disseminated and serious forms of the disease. Hence, certain members of this genus have the capability to disseminate away from the GI tract and establish secondary infections in other tissues by somehow breaching the gut epithelia from the lumen. Therefore, the strategy employed by these pathogens to invade the host via the GI tract

route could be shared by other members of the same genus or clades of mycobacteria (e.g., *M. marinum*) or other enteric pathogens (e.g., *Shigella disenteriae* and *Yersenia pseudotuberculosis*).

A number of enteric pathogens are able to take advantage of the mucosa immune protection of the gut, to cross its protective epithelial barrier and gain access to the viscera and target tissues (Clark et al., 1999). The gut-associated lymphoid tissue (GALT), which is very important as a primary line of defense against invasive microbes of the gut, can also serve as a portal of entry into the body. In humans, the tissue consists of organized lymphoid follicles in the submucosa layer that forms pocket-like structures (i.e., Peyer's patches). Immune cells found in these follicles include macrophages, dendritic cells, and lymphocytes (B and T cells). The patches are separated from the intestinal lumen by specialized epithelial cells (i.e., microfold or M cells) that continuously sample antigens and bacteria from the lumen by phagocytosis and deliver these potential pathogens to basal phagocytic cells for destruction. Hence, after uptake by M cells, these pathogens are internalized by endocytosis, transported to the sub-epithelial layer and delivered to phagocytes for destruction and in turn act as antigen presenting cells (i.e., dendritic cells and macrophages) of the Peyer's patches. Other human pathogens reported to gain access across the gut epithelia via M cells include *Yersenia pseudotuberculosis, Yersenia enterocolitica, Shigella dysenteriea*, *Chlamydia sp*, and reovirus (Momotani et al., 1988). Enterocytes, which are also positioned atop these patches, have been demonstrated as the portal of entry in *M. avium* (Sangari et al., 2001).

1.8. Investigation into spontaneous variation of *M. marinum* type strain

Since *M. marinum*'s isolation by Aronson in the 1920s (see 2.1.1), the bacterium has been propagated in various ways, techniques included passage on glucose media at 30° C or SS media. Repeated passage on media over long periods of time (i.e., decades) increases the risk for the accumulation of point mutations and the possible evolution of derivative strains with varying phenotypic and genotypic characteristics. Spontaneous point mutations occur at a rate of 10^{-10} per base pair replicated (Friedberg et al., 2006). The issue was not resolved until the 1970s with the advent of ultracool freezers (-80° C), making possible the storage of frozen stocks. Additionally, stock cultures for various research facilities acquired *M. marinum* (Aronson) type strain at various times and from different sources. Compounded, these issues may account for accumulated point mutations that could lead to various research facilities having unwittingly acquired different variant strains, all assumed to be genetically identical type strains. These issues present a fundamental obstacle: experiments must be able to be replicated by others.

An example of extensive laboratory acquired variations of the initial *M. marinum* type strain (i.e., *M. marinum* (Aronson)) was noted by Lief Kirsebom and coworkers at Uppsala University in Sweden. The researchers noticed varying growth rates and colony morphologies. Similarly, Pam Small, Univeristy of Tennessee, became the curator of the Trudeau Mycobacterial Collection and noticed morphological differences in the different stocks of *M. marinum* (Aronson). The "rough" (i.e., TMC1218R) variant of the *M.* marinum (Aronson) of this type strain (TMC1218R) has been dispensed by this collection. Indeed, the major U.S.A. stock center, ATCC, has TMC1218R as their type

strain (ATCC927). Subsequent genomic sequencing of these strains revealed a high degree of variation of the purportedly same strain and the concern that very different results depending on which variant one uses. The virulence of TMC1218R (the "wild-type" strain employed in our lab) was previously tested against the *M. marinum* M strain, which has been frozen since its isolation from a human skin infection and presumably never diverged. Results indicate that TMC1218R is as equally virulent as the M strain (Mutoji and Ennis 2012).

1.9. Research statement

Here, I employ the *M. marinum*-medaka infection model to investigate the bacteria passing the fish gut epithelia and to investigate the possibility of inducing protective gut mucosal immunity by a live *M. marinum* vaccine strain candidate. To investigate infection via an oral route, I orally infected fish with fluorescent bacteria to directly observe the bacteria gaining access and colonizing the underlying tissue by surpassing the epithelial layer. Furthermore, I wanted to investigate if vaccinating fish with a live, attenuated *M. marinum* strain would confer mucosal immunity against an orally administered virulent, wild-type challenge. I was also interested in investigating potential differences in virulence between several virulent *M. marinum* type strains obtained from different stock centers.

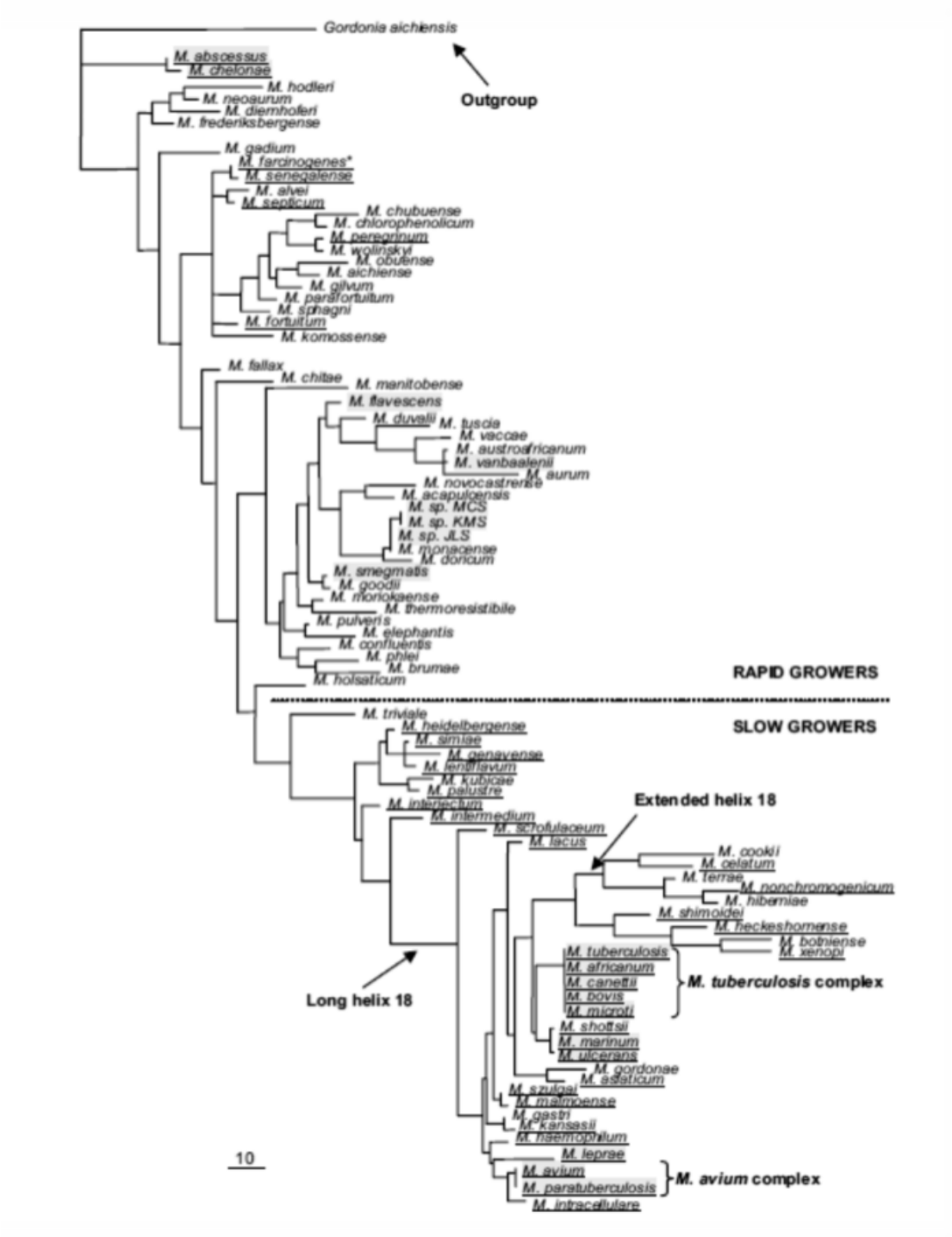


Figure 1.1: Phylogenetic tree of genus *Mycobacterium*. Phylogeny based on 16S rRNA gene sequences of 88 of 130 valid species as seen in a paper published by Pittius et al. (2006). This tree illustrates the separation between the slow and fast growing species.

Mycobacterium Specie	Host (s)	Environmental reservoirs	Disease(s)	Route of infection
M. tuberculosis	Human	None	Pulmonary TB	Aerosols (respiratory tract)
M. bovis	Human, ruminants	None	Pulmonary TB, Intra-abdominal TB, or cervical Lymphadenitis	Respiratory tract gastrointestinal tract
M. ulcerans	Human	Aquatic insects	Buruli ulcer	Insect bites
M. laprae	Human	Possibly armadillos	Leprosy (Hansen's disease)	Respiratory tract Skin to skin contact
M. marinum	Human and poikilotherms (i.e., fish/frogs)	Water(fresh, marine or tap water), soil, protists, biofilm	skin granulomatous infection or Mycobacteriosis	Cuts/abrasions, speculative methods for poikilotherms (ingestion, gills, cuts, etc…)
M. fortuitum	Human, poikilotherms	Water(natural or tap), soil, protists, biofilm	Mycobacteriosis	Respiratory tract cuts/abrasions
M. chelonae	Human, poikilotherms	Water(natural or tap), soil, protists	Mycobacteriosis	cuts/abrasions
M. avium complex (M. avium, M. intracellulare and M. paratuberculosis)	water buffalo, cattle, pigs, deer, horses, and birds	Water(natural or tap), soil, biofilm, dust, food	Lymphadenitis, skin disease, disseminated disease, granuloma enteritis.	Respiratory tract gastrointestinal tract poikilotherms (ingestion, gills, cuts, etc…)

Table 1.1: Mycobacterial species and respective hosts, reservoirs, disease, and route of infection. Listed are a few mycobacterial species, their respective hosts, and routes of infections. Most environmental species exhibit a dual existence as intracellular pathogens and/or free-living organisms.

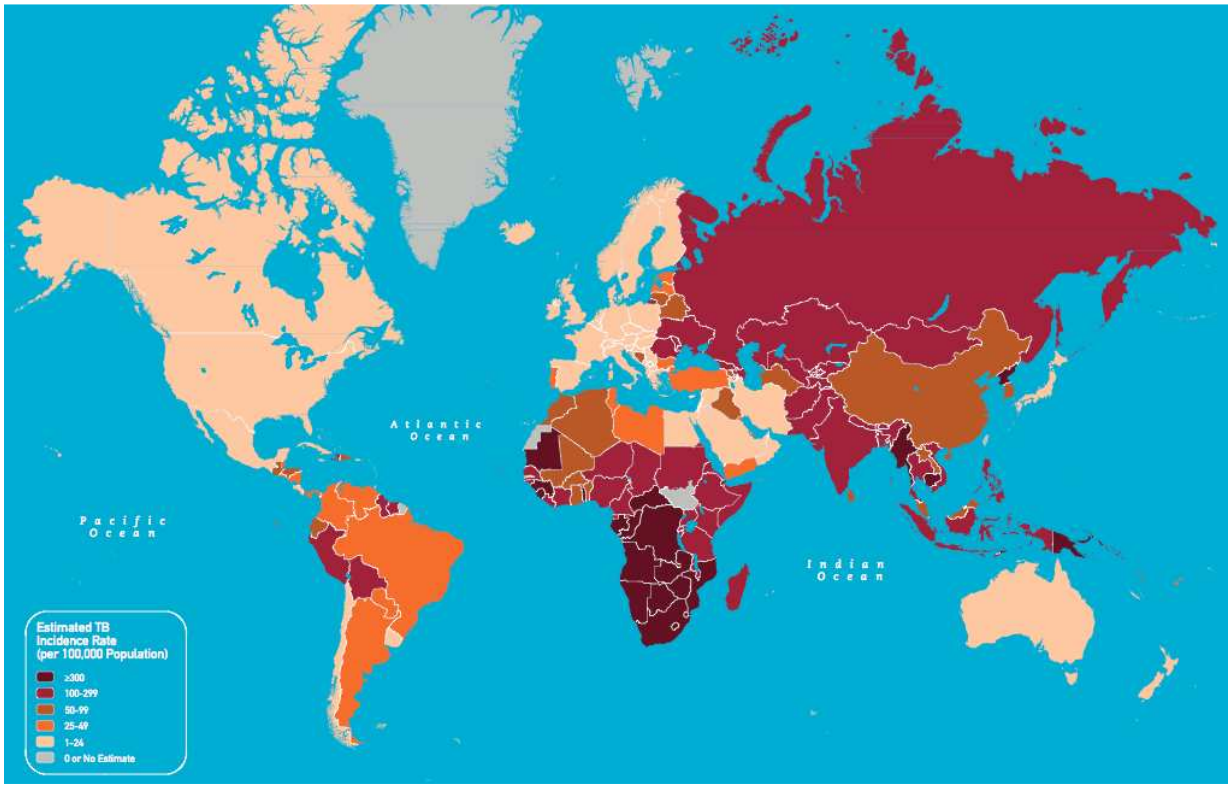


Figure 1.2: Global TB incidence. A map showing the global distribution cases as reported by the World Health Organization (2012). This map shows all estimated incidences of TB per 100,000 inhabitants.

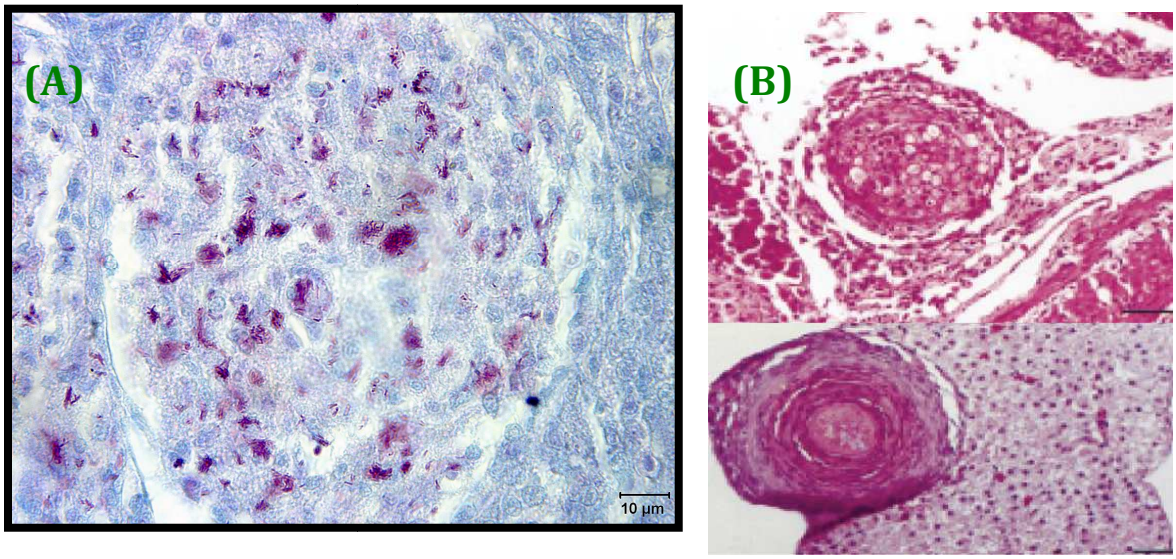


Figure 1.3: Histology of granuloma sections. The images show multiple granulomas. (A) reveals acid-fast rods of sections of a granuloma isolated from medaka (Mutoji, 2011). (B) shows granulomas isolated from goldfish (Talaat et al., 1998).

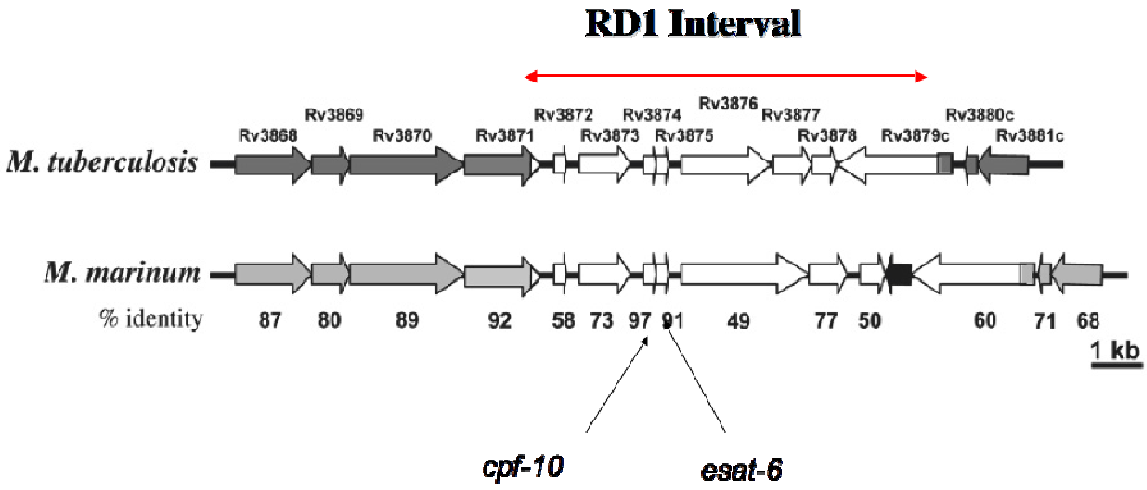


Figure 1.4: RD1 interval. Representation of RD1 interval in *M. tuberculosis* and *M. marinum* diagramed by Volkman et al. (2004). The CPF-10 and ESAT-6 genes (black arrows) are known virulence genes with a high degree of amino acid conservation between both species (97% and 91%, respectively).

Figure 1.5: Global distribution of BCG administration. The map shows the global distribution of BCG vaccine administration to infants.

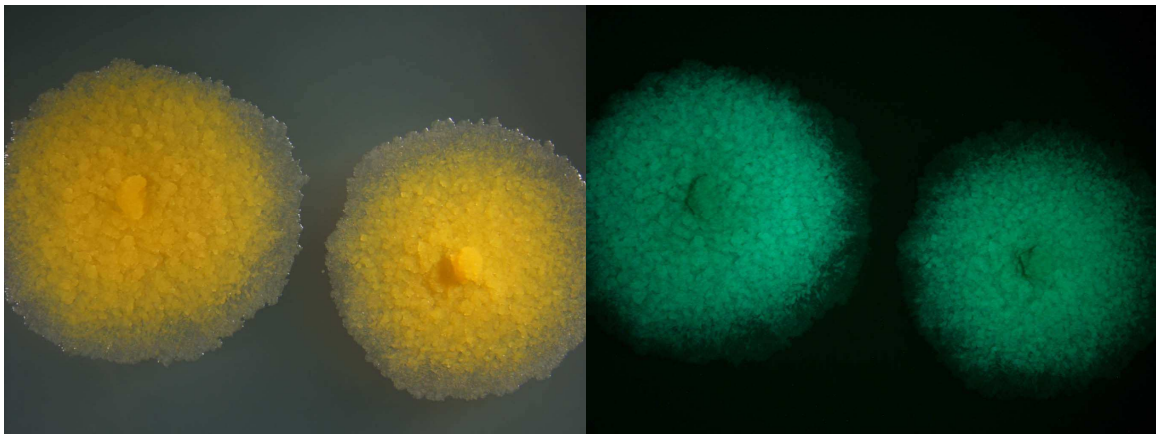


Figure 1.6: *M. marinum* colonies. Photographs of *gfp*-expressing *M. marinum* colonies in brightfield and green fluorescence (Mutoji, 2011).

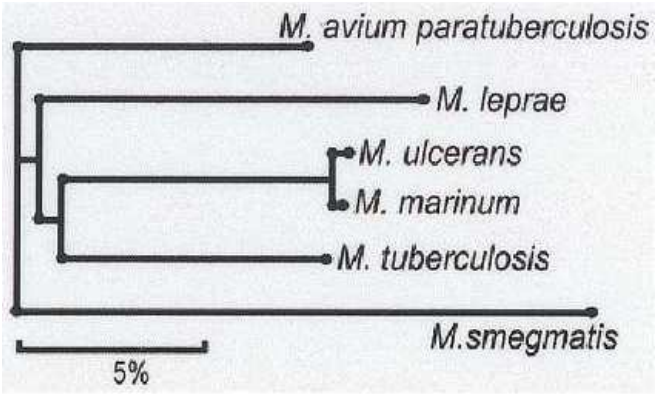


Figure 1.7: Whole genome analysis phylogeny construction. Phylogenetic tree based on whole genome analysis reported by Stinear et al. (2008). *M. marinum* is the closest relative to the *M. tuberculosis* complex.

Chapter 2

Materials and Methods

2.1 Materials and Methods pertaining to all result chapters

2.1.1. Bacterial strains and cultures

Strains were frozen and stored at -80° C in 30% glycerol until needed. *Mycobacterium marinum* strains were cultured in Middlebrook 7H9 (M7H9) liquid media supplemented with 10% albumin dextrose salt (ADS), 100 µg/ml cycloheximide, 0.2% tween 80, and 0.5% glycerol at 28° C with shaking. The volume of prepared culture varied from 5 mL grown in test tube to 100 mL grown in 250 mL bottles based on specific need of a particular experiment (Broussard and Ennis, 2007). Strains were diluted and plated for viability count on Middlebrook 7H10 agar (M7H10) supplemented with 10% ADS, cycloheximide (100 µg/mL) and 0.5% glycerol incubated at 30° C.

All strains used in experiments are listed in Table 2.1.

2.1.2. Medaka aquaculture

All fish used for these experiments were from a transgenic line of Japanese medaka (*Oryzias latipes*). The medaka transgenic line originates from Dr. Richard Winn (2000), University of Georgia, the fish were propagated to develop on-site colonies at the University of Louisiana (Broussard and Ennis, 2007). Fish were housed in ten-gallon aquaria, reaching a maximum density of 30-50 adult fish per tank. Tanks were regulated at 28° C with recirculating filtration. Filters were refreshed once a month. The husbandry facility employed a regulated photoperiod to mimic sunlight during the fish's reproductive season—16 hours light, eight hours dark. Twenty percent water changes

were performed bimonthly. Water changes are necessary to remove toxic metabolic wastes, such as ammonium. Fish were fed three times daily with Otohime, brine shrimp, and Aquatic Systems high protein flakes constituting the meals.

A day prior to exposure, four to six month old fish were transferred to the Biosafety Level 2 (BSL-2) infection lab. The infection lab was kept on the same photoperiod and at the same temperature as the husbandry lab. Fish were individually kept in 0.5-liter containers for inoculation, for one week. Following inoculation, fish are transferred to 5.7-liter containers which can hold five to 15 fish. Water changes were performed weekly.

2.1.3. Preparation of infective doses

Cultures of *M. marinum* were grown and then collected at an optical density at 600 nm (OD) of 0.5 to 1.0. Bacterial cells were pelleted by centrifugation (6,000 rpm, 10 min., 4° C) and the supernatant was discarded. The bacterial pellet was resuspended into appropriate volumes of PBS. Bacterial concentrations in solutions used for infective doses are approximately 10^8 bacteria per mL of PBS.

When infecting fish with mixed cultures (e.g., competition experiments), both strains were grown-up separately. The cultures were collected at similar optical densities and resuspended into appropriate volumes of PBS to achieve equal densities. To determine input dosages, appropriate dilutions of culture were plated on M7H10 plates to quantify colony forming units.

2.1.4. Oral infections via larval vectors

M. marinum is delivered to the fish using mosquito larvae as a vector. Mosquito larvae raised from zygotes that were deposited into a beaker with a solution of cultured

M. marinum/PBS solution (OD_{600} 0.5-1.0 for 18-24 hours) for a final concentration of ~10^8 bacteria/mL solution. Infection meals consisted of five mosquito larvae per fish (Mutoji, 2011). Fish were fed four infection meals, one every other day. The mosquito larvae were then retrieved from a beaker with mycobacteria and rinsed to remove external bacterial cells. Five mosquito larvae (one "meal") were then placed into a beaker housing one experimental fish. For infection feedings, fish were kept in individual containers to ensure the same dosage for each fish. The fish readily consumed mosquito larvae. Inoculating dosages were determined by viable cell counts from mascerated control larvae that were not used to infect fish. Viable cell counts were determined by plating colony forming units at appropriate dilutions on M7H10 plates supplemented with glycerol (0.5%), cyclohexamide (100 µg/ml), ampicillin, polymixin B sulfate, and the selective antibiotic (kanamycin or hygromycin), if needed.

2.1.5. *M. marinum* infections via IP injection

Fish were infected via intraperitoneal (IP) injection of *M. marinum* under different conditions. As per methods described by Broussard and Ennis (2007), OD_{600} = 0.6 culture was diluted to appropriate density, and 20 µl of this suspension was the volume used to inoculate fish. Appropriate dilutions were plated to determine infection dosage of the IP injection.

2.1.6. Mosquito larvae

Yellow Fever mosquito (*Aedes aegyti*) larvae were used as an intermediate host or vessel to inoculate fish with *M. marinum*. *A. aegypti* embryos were purchased from Benzon Research (Carlisle, PA). As per vendor's instructions, beakers containing water from aquaria in the fish husbandry lab tanks were placed in a water bath, operating at

29° C. Crushed fish flakes were added to the aquarium water to promote bacterial growth that yield anoxic water, which assists in the synchronizing of hatching. 24 hours later, the mosquito larvae fertilized eggs were added to the beaker. Typically, three days later, mosquito larvae hatched and reached developmental stage instar three or four (Bar and Andrew, 2013). If needed, the larval diet of finely ground fish flakes was kept to a minimum to prevent rapid larval development.

2.1.7. Dissection and determination of bacterial loads in infected fish organs

To determine bacterial loads of *M. marinum* of target organs, experimentally-infected fish were sacrificed four to 14 weeks post infection. The length of time elapsed between infection and dissection depended on the nature of the experiment. Fish were humanely euthanized by an established protocol (Broussard, 2007) essentially by an "over-dose" of anesthesia, a 0.1% solution of MS-222 (Tricaine). A ventrile incision was performed with sterile implements to expose the viscera and remove the liver and kidney of each fish. All livers and kidneys were homogenized into 1 mL of sterile PBS, and ten fold dilutions were performed. Appropriate dilutions were plated on M7H plates supplemented with cyclohexmide, polymixyn B, glycerol, amplicillin. Plates were then incubated at 30° C for approximately ten to 14 days. The number of viable cell counts in each organ was determined by performing colony counts (i.e., CFU), and the average number of CFU was used to calculate the bacterial load per organ. Prior to colony count, plates were left by the window exposed to natural light for close to two days for development of the diagnostic yellow pigments in *M. marinum* colonies.

2.1.8. Plating for viable count of fluorescent colonies of *M. marinum*

Most of microscopy employed utilized a NikonSMZ800 (Nikon) Stereoscopic Microscope equipped with X-Cite 120 for fluorescence illumination and filters fitted for detection of bacteria carrying either green fluorescent protein (*gfp*) or red fluorescent protein (*rfp*). Colonies were examined for expression of the correct fluorescence (red or green), as were mosquito larvae fed with *gfp* or *rfp* expressing bacteria.

2.1.9. Preparation of gut samples for histochemistry and quantitative reverse transcriptase polymerase chain reaction (qRTPCR)

Gut samples were prepared for histochemistry to be analyzed by Irene Salinas at the University of New Mexico. Infected fish were sacrificed at desired time points, and the gut was dissected out of these animals. Subsequently, the dissected gut was opened by a lateral insertion from the anterior to the posterior ends. These opened digestive tracts were then rolled onto itself from the anterior end to the posterior end. Once rolled, the dissected samples were inserted into a well containing Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, N.C.) and stored in -80° C until shipped. Samples were shipped overnight with dry ice to ensure the samples remained frozen.

For qRTPCR sample preparation, the gut was dissected out of the fish. All instruments used for dissection were treated with RNase EZ to remove RNases. Samples were stored in RNA stabilizer and frozen in -80° C freezer until shipped. Samples were shipped overnight with dry ice to ensure the samples remained frozen.

Irene Salinas prepared all dissected and frozen gut samples for histology, fluorescence microscopy, and qRTPCR analyses.

2.2. Vaccine experimental methods

2.2.1. Experimental timeline of vaccination studies

All doses to fish, vaccine and wild-type challenge strains, were given four meals of five infected larvae per meal. Fish exposed only to the vaccine candidate strain were sacrificed three months post exposure. Fish exposed only to the wild-type *M. marinum* challenge were sacrificed two months after the wild-type challenge. Vaccinated fish were challenged a month after vaccination and sacrificed two months post-challenge. An additional group received a second booster dosage of the vaccine candidate a month after being initially vaccinated. The "boosted" fish are then challenged two weeks after exposure to the "boost."

2.2.2. Data statistical analysis

To determine if vaccination conferred statistically significant levels of protection against an oral, wild-type challenge, a permutation test was performed on the data. The program that performed the statistical analyses is the statistical package R and carried out by collaborator Dr. Luis Novelo from the University of Lousiana. The permutation test was applied with transformed data ($x = \ln(x + 1)$). The transformed data sets compared the vaccinated-only fish (n= 25) and the vaccinated-challenged fish (n= 27). To begin, a T(observed) was calculated: the sample mean of the transformed vaccinated-challenged data (i.e., treatment group) minus the sample mean of the transformed vaccine-only data (i.e., control group). Theoretically, this data should be large to indicate a substantial difference in mean between the two sample groups (i.e., null hypothesis: T(observed)= 0). Once T(observed) is calculated, the program randomly reorganizes the data, but retains the groups' original labels and sample sizes,

importantly. The new data sets are then compared in the same manner as T(observed), by subtracting the new sample mean of the vaccinated-challenged data by the new sample mean of the vaccinated-only data (i.e., T(permutation)). The theory of the permutation test states that T(permutation) should be consistently, considerably smaller than T(observed) if the treatment was successful. The test concludes the probability that the treatment (i.e., vaccination) did not have an effect: the probability of observing a value as extreme or more extreme than the actual value observed is manifested by the p-value. 10,000 permutation tests were carried out on the data to obtain p-values.

2.3. Investigation into spontaneous variation of *M. marinum* type strain experimental methods

2.3.1. Strains used

Competition experiments were performed to directly compare the virulence of TMC1218R or DE4373 (derived from *M. marinum* Aronson) to other *M. marinum* wild-type strains used in laboratories around the globe. For the summary of strains, see table 2.1 and section 1.11. DE4578-4583 contained pMH94 plasmid, which integrates into the chromosome at the L5 *attB* site of the chromosome. The plasmid confers kanamycin resistance.

2.3.2. Delivery of *M. marinum* to fish by mosquito larval vessel

Strains were grown separately as discussed above. When infecting with a mixed culture comprised of two strains, the cell concentration of both strains were collected at approximately the same OD_{600} of 0.5 and resuspended in PBS at approximately the same concentration so as to obtain a 1 to 1 ratio. The bacteria were delivered to the fish

using mosquito larvae as an intermediate, as previously described. Competition experiments allow researchers to compare virulence of two strains within a controlled environment (i.e., the same immune system response). Putting both strains into the same host challenges both strains to an identical immune response.

2.3.3. Calculating competition index

The competitive index (CI) of each fish was calculated as previously described by Ruley et al. with the following equation: [(CFU mutant output)/(CFU wild-type output)]/[(CFU mutant input)/(CFU wild-type input)]. For two stains that are equivalent in virulence and organ colonization, one would expect a CI value at approximately one. Mutant strains with reduced virulence would have CI values less than unity (i.e., <1). Strains were differentiated based on presence or lack of *rfp* expression in bacterial colonies.

Strain	Specie	Relevant trait	Source
DE4373	*M. marinum*	TMC1218R L5::pDEAM2 (rfp, kan ^{r})	Laboratory collection
DE4374	*M. marinum*	TMC1218R L5::pDEAM1 (gfp, kan^{r})	Laboratory collection
DE4558	*M. marinum*	M strain with RD1 locus deletion	Lalita Ramakrishnan, University of Washington
DE4578	*M. marinum*	DSM44344T L5::pMH94 (kan^{r})	Leif Keirsebom, University of Uppsala
DE4579	*M. marinum*	DSM43518 L5::pMH94 (kan^{r})	Leif Keirsebom, University of Uppsala
DE4580	*M. marinum*	NTCC20998 L5::pMH94 (kan^{r})	Leif Keirsebom, University of Uppsala
DE4581	*M. marinum*	TMC1218S L5::pMH94 (kan^{r})	Leif Keirsebom, University of Uppsala
DE4582	*M. marinum*	DSM43519 L5::pMH94 (kan^{r})	Leif Keirsebom, University of Uppsala
DE4583	*M. marinum*	NTCC2275 L5::pMH94 (kan^{r})	Leif Keirsebom, University of Uppsala

Table 2.1: Strains used in experiments. List of all bacterial strains used in experiments.

Chapter 3

Results

3.1 Transmission of *M. marinum* in medaka

3.1.1. Establishment of *M. marinum* infection in medaka via oral exposure

Control experiments were conducted to ensure comparable disease presentation (i.e., establishment of life-long, chronic infection) by oral route infections, as seen when fish were infected via intra-peritoneal (IP) injection. In this experimental design, a cohort of fish was infected via the oral route method utilizing mosquito larvae, as a carrier for *M. marinum*. Several weeks post-infection, fish were sacrificed and dissected organs were homogenized and plated for CFU count to quantify bacterial burdens in fish exposed to the pathogen. Orally infected fish were found to have heavily colonized kidneys and livers under similar time frame, as with IP-infections (data not shown). The similarities in target organ colonization, organ bacterial burdens, and infection time course suggest that IP and oral infections share similar disease progression from initial exposure to establishment of infection in target tissues. These results were consistent with results previously described by Mutoji (2011). Similar disease progression by these two routes would suggest that the bacteria delivered to the lumen of the fish gut does not delay infections, that instead the pathogens are able to rapidly cross the epithelial layer of the digestive tract and infect the host systemically.

Additionally, the experimental approach employed modified the method described by Mutoji (2011) to feed bacteria to the mosquito larvae. Previously, a large 100 mL of culture was grown-up to feed to mosquito larvae. The variation I employed was to grow 5 mL cultures and then combine the cultures into a 20 mL solution. The

reduction in volume was carried out to speed up the time needed to grow up appropriate culture densities and minimize contamination risks. The results show that a lower dosage (i.e., 20 mL) is sufficient to fill mosquito larvae with bacteria to deliver to fish and infect fish (see below).

3.1.2. Investigations into *M. marinum* crossing the epithelia and establishing infection in underlying tissues

In these experiments, I wanted to investigate the passage of *M. marinum* from the lumen of the gut across the epithelial layer of the gut and investigate if the pathogen was observable associated to the villi and underlying tissues, such as the lamina propria or submucosa, and to get an idea of the time required for the bacteria to cross the epithelia. According to Broussard and Ennis (2007), using IP injections of *M. marinum* to infect medaka, the pathogen is able to spread and establish infections in target organs away form the site of injection. As observed in other vertebrates, the IP-injected pathogen is presumed to be transported from the site of infection by resident peritoneal phagocytes via the vascular system to the target organs.

Therefore, I wanted to gain insights into the mechanism for *M. marinum* passing from the lumen to underlying gut tissues. Figure 3.1 diagrams the anatomy of a typical human gut mucosal anatomy, which is similar to the anatomy of a fish gut. To determine if the pathogen was able to cross the epithelial layer of the gut, fish were infected with TMC1218R, derived from type strain *M. marinum* (Aronson), carrying an integrated *gfp* construct (pDEAM1). The fluorescent marker carried by the bacteria assists in the tracking of the bacteria in tissues. Orally infected fish were sacrificed after one or four weeks post-infection, denied food for three days to help clear the gut contents, and

then, guts were dissected from euthanized fish. Frozen sections of the dissected gut were performed followed by histology and fluorescence microscopy by our collaborator Irene Salinas at the University of New Mexico. The epithelial layer of the gut can be visualized from DAPI stained nuclei. As shown in Figure 3.2, fluorescent bacteria were present in the gut and principally found attached to the apical tips of the villi. Also, aggregates of fluorescent bacteria (i.e., infection foci) were documented by our collaborator in the vascularized lamina propria below the epithelial layer (Figure 3.1), which presents a region for the pathogen to come in contact with host immune system cells, such as surveying macrophages. Furthermore, fluorescent infections foci were observed by our collaborator in the mucosa and submucosa in as little as one week post-exposure to the pathogen.

These results indicate that *M. marinum* is able to bind to the tips of the villi, and can in turn transit to underlying host tissues and establish internal infection in less than one week. These studies also confirm that orally infected fish continue to carry *M. marinum* in the gut for months. This persistence of mycobacteria in the gut lumen is consistent with what was described by Mutoji (2011) where some individuals were found to carry acid-fast rods 11 weeks following oral infection by larvae. Here again, fluorescent bacteria were observed within the lumen, but many appear to be specifically attached to the tips of the villi.

3.1.3. Shedding

Since *M. marinum* can be seen still present in the gut lumen a month or longer post-infection, I speculated that some bacteria may be continuously shed into the surrounding media by the host. Experiments were carried out to detect *M. marinum*

shed into the surrounding media by infected fish. *M. marinum* was successfully cultured from the water at one week and three weeks post-infection in containers housing infected fish. Titers found were between zero to ten CFUs per mL of water and confirms previous preliminary observations in our lab (Mutoji, 2011; Root, 2012). This suggests that fish are actively shedding the bacteria into the water, which may be a route for pathogen transmission to cohabitating fish and is consisted with horizontal transmission between fish by Mutoji (2011) and Root (2012).

3.2. Vaccine Studies

3.2.1. Vaccination experiment

As noted above, *M. marinum* infects medaka by initially infecting the tissues in and under the epithelial layer of the digestive tract. We presume the bacteria are transmitted to target organs by phagocytes. It is now well established that a majority of the vertebrate immune response is associated with gut mucosa, called Gastro-Intestinal Associated Lymphoid Tissue (GALT) and in response to a new bacterial agent, will induce a mucosal immune response to that agent. Here, I wanted to investigate the possibility of inducing mucosal immunity against *M. marinum* in medaka by first, priming fish with a live, mutant *M. marinum* strain that has been shown to be essentially avirulent, followed by a challenge of the wild-type, virulent strain. The candidate vaccine strain used is an *M. marinum* strain carrying an engineered deletion in the "RD1 locus." This deletion removes several genes, like ESAT-6 (Figure 1.4), which are known to have important roles in virulence and a similar deletion is carried in the BCG vaccine strain. Liver and kidney bacterial burdens of the wild-type *M. marinum* strain were used as end points to monitor whether the candidate vaccine strain was protective to an

infectious challenge. Fish were divided into three experimental groups: the first group of fish was only exposed to the virulent, wild-type *M. marinum* strain, here, carrying the *rfp* reporter, without any benefit from prior exposure to the candidate vaccine strain. These fish were sacrificed two months post-infection, and the target organs were examined for burdens of the fluorescent-marked CFU. The second group of fish was exposed to the vaccine candidate only, and these fish were sacrificed three months post-infection to check for organ colonization by the unmarked vaccine strain. The third group was initially exposed to the candidate vaccine strain; then, a month later, these fish were given an oral challenge by the virulent wild-type *M. marinum* strain. The fish from groups one and three were sacrificed two months after the challenge exposure and fish exposed only to the vaccine (i.e., group 2) were sacrificed three months after exposure to the vaccine strain. Target organs were dissected, homogenized, and plated for CFU counts.

Of the 25 fish exposed solely to the wild-type, challenge strain (group 1), 22 fish were culture-positive (~88%). Fish that were vaccinated and subsequently challenged (group 3) were culture positive for wild-type strain at a ~40% frequency (11/27 fish), suggesting some protection by the vaccine strain. Interestingly, just one fish exposed only to the vaccine candidate (group 2) was culture-positive or a ~5% chance of the attenuated strain persisting in the host (1/23 fish) (Figure 3.3), which indicates the ΔRD1 mutant is effectively cleared by the fish immune response at three months post-inoculation.

As noted above, the proportion of animals that were culture-positive dropped from 88% in the challenge control to 40% in the vaccinated-challenge experimental

group; significantly, the organ bacterial burdens of the *rfp*-marked challenge strain were dramatically reduced with vaccination. Comparing kidney bacterial burdens, vaccinated fish (n= 27) averaged a nearly 1,700-fold lower *M. marinum* infection burdens than unvaccinated fish (n= 25). Comparing median liver bacterial burdens, fish exposed to the vaccine candidate (i.e., vaccine-only, vaccine-and-challenge, and boost groups) had 130-fold decline in infection burden. Figures 3.3, 3.4, and 3.5 highlight the differences in the abundance of mycobacteria colonizing organs between vaccine-plus-challenge fish and challenge-only fish for these organs. Figures 3.6 to 3.9 show histochemistry results comparing vaccinated and unvaccinated fish. Interestingly, only one field of view had red-fluorescent foci of infections was found (Figure 3.9) in the gut of the vaccinated fish.

A permutation test was employed on the data to evaluate statistical significance of the differences observed between the challenge-only group and vaccinated-challenged group. This analysis further supports the hypothesis that the vaccination provides protection against a wild-type challenge. The p-value for livers data was calculated at 0.00006; and for kidneys, the p value was calculated at 0.00031. These analyses would confirm that oral vaccination by the ΔRD1 mutant strain confers significant protection in the host against a wild-type, virulent challenge. In addition, three months post-exposure, the vaccine strain usually does not persist. Except for one in 46 dissected organs, the bacterium was cleared by the host immune system. Moreover, mucosal immunity is likely at the gut mucosal level, since only one foci of infection was observed in epithelia of the vaccinated-challenged fish.

3.2.2. Test to determine if vaccine boosters enhance protection in medaka

To investigate if the protective immunity by the vaccine strain could be enhanced, I conducted additional experiments that included additional immunizations. A group of fish received a second round of exposures to the vaccine candidate, referred to here as a "booster." The booster dosage consisted of two meals of five mosquito larvae per fish spaced-out over three days (i.e., half of previous vaccination dosage) given a month after the initial vaccine candidate priming. Fish that received the booster dosage were later exposed to the same *rfp*-marked wild-type *M. marinum* challenge strain two weeks post-boost administration. At six weeks post-challenge, fish were sacrificed and tested for kidney and liver colonization. Five of the nine fish (~55%) were found to be culture-positive for the wild-type, virulent challenge strain; though, the burdens of *M. marinum* were low. Due to the small number of animals used, it is unclear if the boost offered protection, and if burdens in these animals were significantly different than fish exposed to the candidate vaccine strain regimen (i.e., vaccine-challenge) (Figure 3.10). In retrospect, the booster dosage may have offered little additional protection since microscopy studies of intact infected medaka have shown that acid-fast rods are present in the gut at 11 weeks post-exposure (Mutoji, 2011). Furthermore, microscopy studies carried out with Dr. Salinas show the bacteria to be in the gut at four weeks post-infection.

3.2.3. Test to determine if half dosage of vaccine induces protection against a wild-type challenge

To investigate the protective quality of the strain using lower vaccine doses, I orally infected fish by two feedings using the ΔRD1 mutant strain. I subsequently

challenged the fish orally with the wild-type strain four weeks after exposure to the vaccine. In this experiment, I wanted to know if exposure to the vaccine candidate in smaller doses was also sufficient to confer protective immunity.

The results obtained suggest that two meals of the vaccine strain confer some protection against an oral wild-type challenge. Though 4 of the 5 fish were culture positive, burdens were relatively low (Figure 3.11). These preliminary results suggest that, while highly attenuated, the ΔRD1 mutant appears to cross the epithelia readily and appears to elicit a strong immune response. Increasing the samples size of fish would be important to confirm if protective immunity can be conferred by a smaller vaccine dose.

3.2.4. Investigation into compartmentalization of protection induced by exposure to candidate vaccine strain

Furthermore, I wanted to explore if the protective immune responses against a wild-type challenge were confined to the gastrointestinal tract (i.e., mucosal immunity). Ifimmunity were confined to the mucosal surfaces then one would expect no protection would be conferred to IP-injected challenge (Wilson et al., 2011). I vaccinated fish orally with the ΔRD1 mutant; four weeks after, I challenged the fish with an intra-peritoneal injection of wild-type *M. marinum*.

Of the fish that were vaccinated orally and subsequently challenged via IP injection, 6 of the 6 fish were culture-positive, compared to only 40% infection frequency when fish were orally vaccinated and orally challenged (Figure 3.12). These results suggest that protection against a wild-type challenge is compartmentalized to

the gut. Though, subsequent experiments will have to increase sample size to further investigate these findings.

3.2.5. Investigating clearance of the candidate vaccine strain in medaka organs

I described above, the vaccine strain candidate was cleared in all but one test animal three months post-exposure. To further investigate the persistence or clearance of the ΔRD1 mutant strain fish were inoculated, and then, target organs were dissected out a month after exposure, rather than three months. Here, the vaccine candidate is present one month post-exposure (3/6 fish were culture positive), albeit the bacterial burdens were notably low (1-9 CFU of *M. marinum*) (Figure 3.13). We speculate that the vaccine strain is being actively cleared over time and that at one month a remnant of this strain remains. We also suggest that at later time-points (e.g., three months), greater clearance would occur since at three months post-vaccination, only one in 25 fish (or one in 46 dissected organs) were found to carry a low burden of the ΔRD1 mutant (Figure 3.14).

3.2.6. Quantitative reverse transcriptase PCR of vaccinated gut samples

To investigate the host immune response to the attenuated *M. marinum* strain, I exposed medaka to the vaccine candidate. A month after exposure, the entire gut was dissected out of the fish, and these samples were sent to our collaborator Irene Salinas, whose lab compared the mRNA levels for the IgM and CD8 genes in the vaccinated versus unvaccinated gut tissues. Preliminary results show that there is a seven-fold upregulation for IgM and five-fold upregulation for CD8 in vaccinated gut tissue (Figure 3.15).

3.3. Investiongation into spontaneous variation of Mycobacterium marinum type strain from different stock centers

In these experiments, I wanted to investigate any differences in virulence between seven strains of *M. marinum* derived from the original type strain *M. marinum* (Aronson), which are purported to be identical but have been found to be genotypically and phenotypically different. The whole genome for each variant was determined, revealing a significant number of point mutations and a few deletions (Kirsenbom, personal communication). Fish were infected as indicated in section 2.4. Fish were sacrificed six weeks post-infection and organ samples were plated to determine bacterial burden in fish.

Clear morphological differences are visible when comparing the strains, such as colony size, pigmentation, and texture. Preliminary competition infections suggest differences in virulence between the competing strains. According to average CI values, *M marinum* DSM44344T is <10-fold to <100-fold less virulent than TMC1218R. DSM43518 was found to be <10-fold to <1000-fold less virulent than the wild-type TMC1218R strain. No fish infections were recorded during the DSM43519/TMC1218R competition experiment. NTCC20998 was preliminarily found to be <10-fold to <100-fold less virulent than TMC1218R. TMC1218S appears to be <2-fold to <10-fold less virulent than TMC1218R. NTCC2275 appears to be >100-fold more virulent than the TMC1218R wild-type. Further tests are under way to better quantify these differences in virulence.

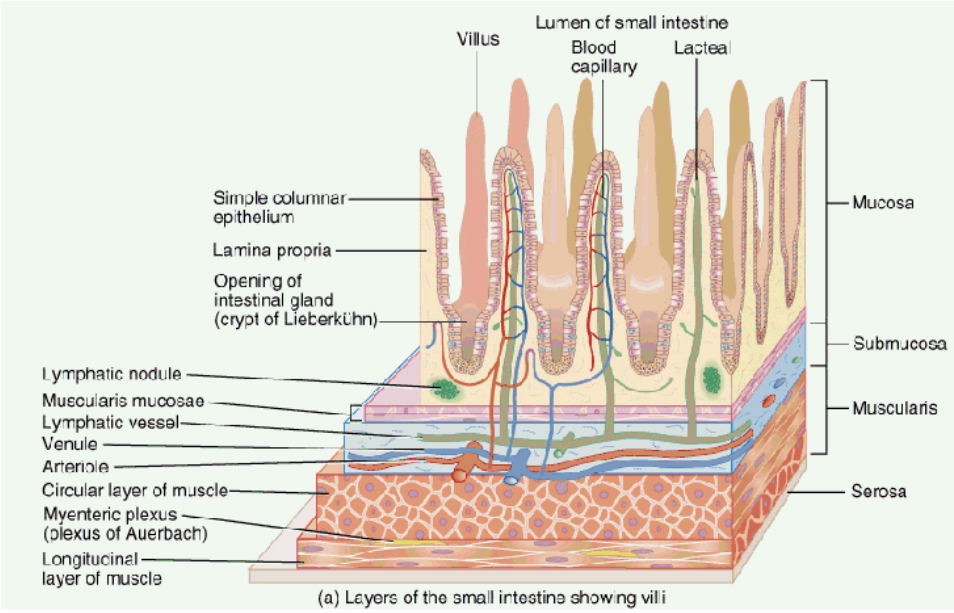


Figure 3.1: Stylized diagram of gastro-intestinal tract anatomy. The villi represent the luminal interface. The lamina propria is the first vascularized region that invading bacteria would encounter. Once crossing the epithelial layer of the villus, the bacteria would be expected to be engulfed by phagocytic immune cells and transported from underlying tissues to the target organs. (http://faculty.southwest.tn.edu/jiwilliams/models_of_the_digestive_system.htm).

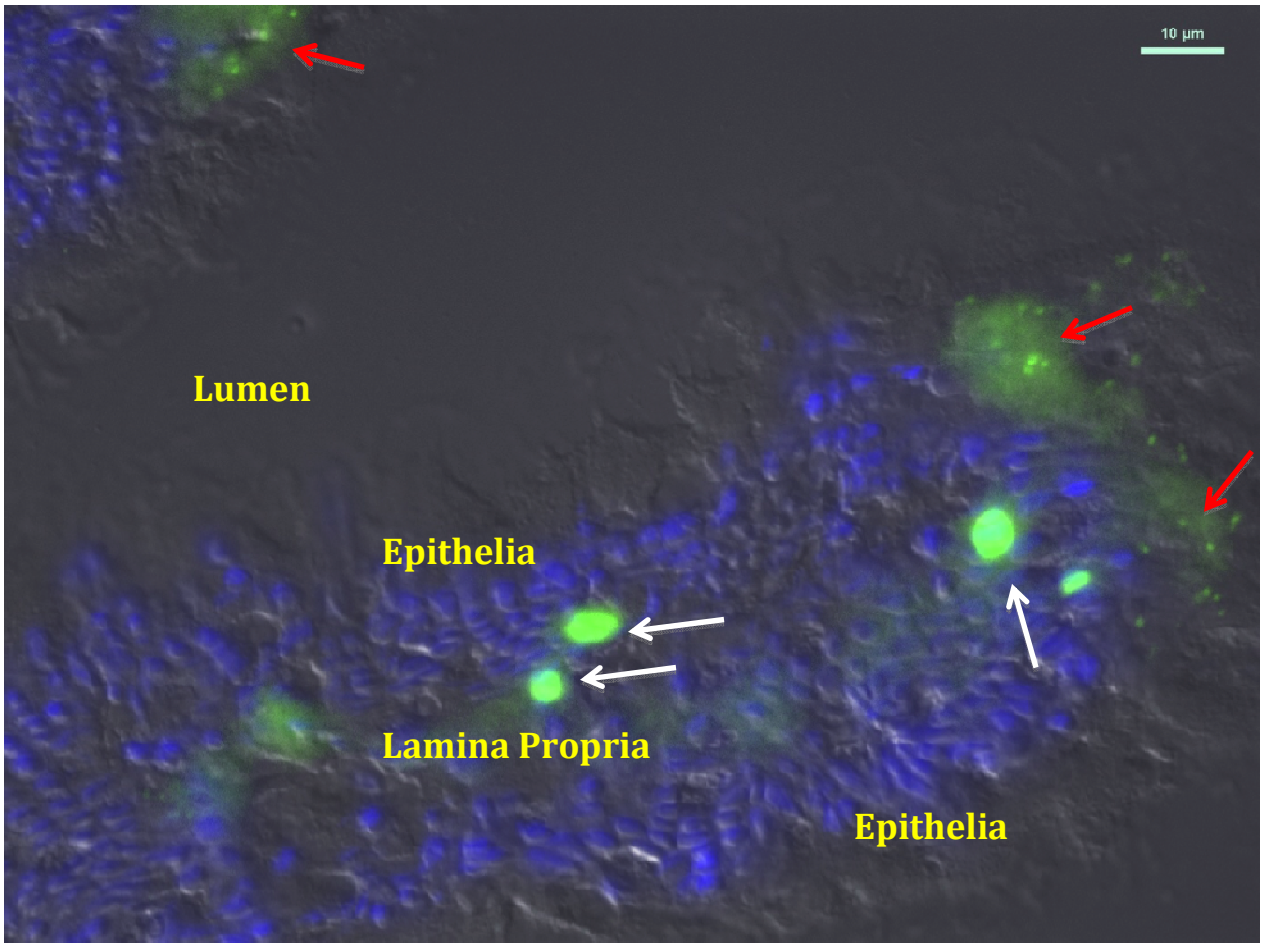
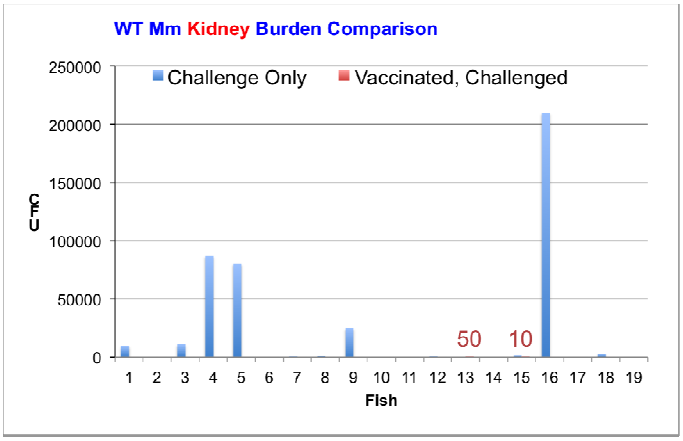
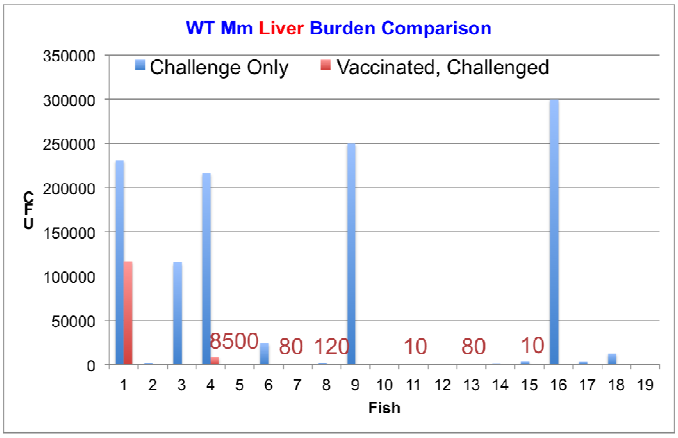


Figure 3.2: Fluorescence image of a frozen cross section of medaka gut four weeks post-infection by *gfp*-expressing *M. marinum* carried in mosquito larvae. The epithelial layer of the gut is indicated by the DAPI-stained blue nuclei of the host cells. The rods are observed (red arrows) attached to the apical portions of the gut villi. Foci of infections are observable in lamina propria tissue of villi (white arrows). Image provided by Irene Salinas.

(A)



(B)



(C)

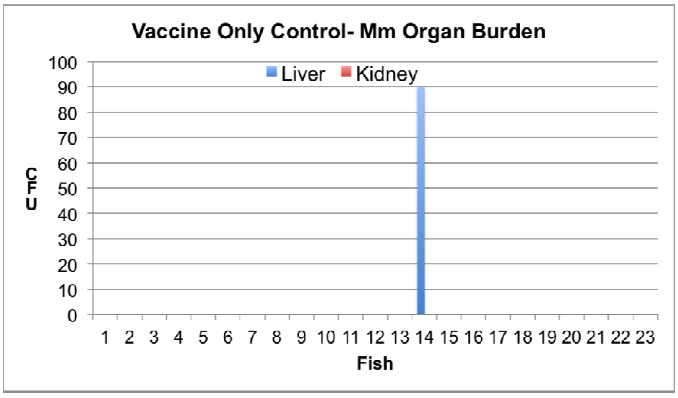


Figure 3.3: Vaccination significantly protects against challenge. (A) shows the relative wild-type *M. marinum* burdens in the kidneys, comparing vaccinated-challenge to challenge-only fish. (B) shows the relative wild-type *M. marinum* burdens in the liver. (C) shows that the RD1 mutant is readily cleared by the medaka immune system.

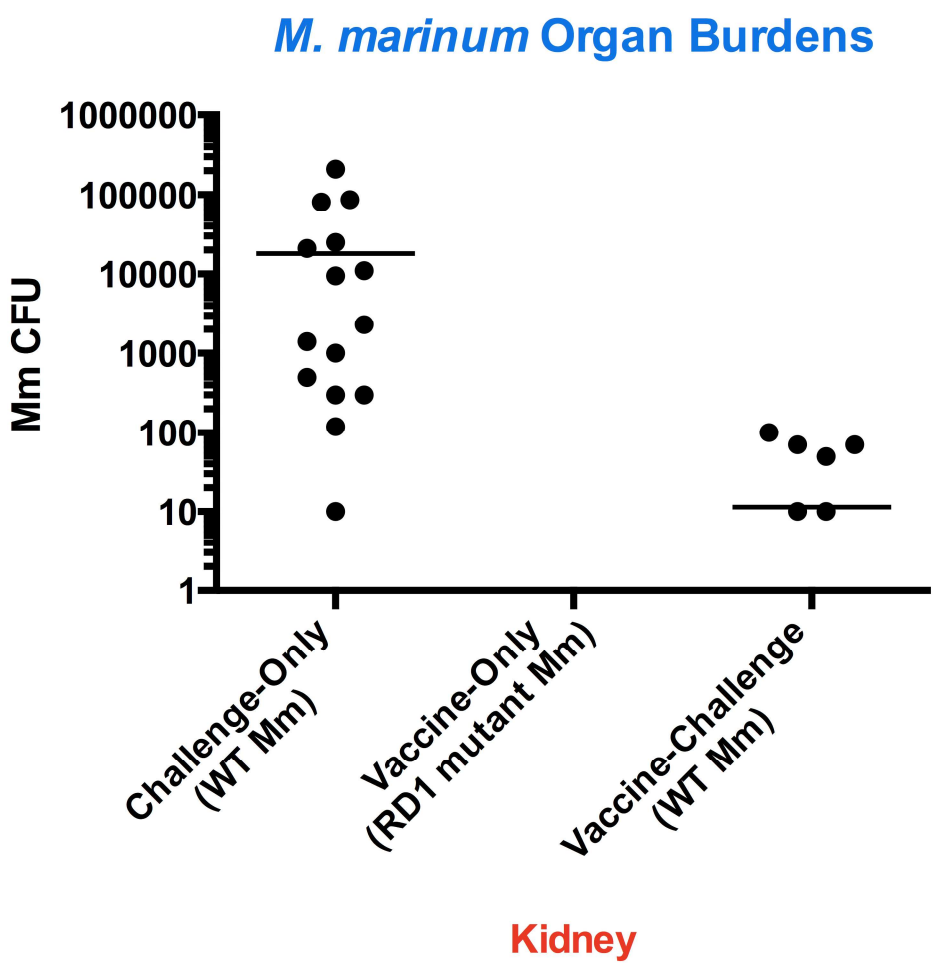


Figure 3.4: Protective effects of vaccination on organ burdens. Kidney burdens in individual fish are comparing initially vaccinated and then challenged by the *rfp*-marked wild-type strain (DE4373) to burdens of the ΔRD1 mutant (DE4558) in fish that only received the vaccination to the wild-type burdens in fish which did no benefit from vaccination. Black bars represent the statistical average of each group. Dots represent individual burdens. Not shown are all "zeros." All culture-negative "zero" results correspond to <10 CFU per organ. This graphical representation of these data was produced by using the Prism 6 statistical analysis program.

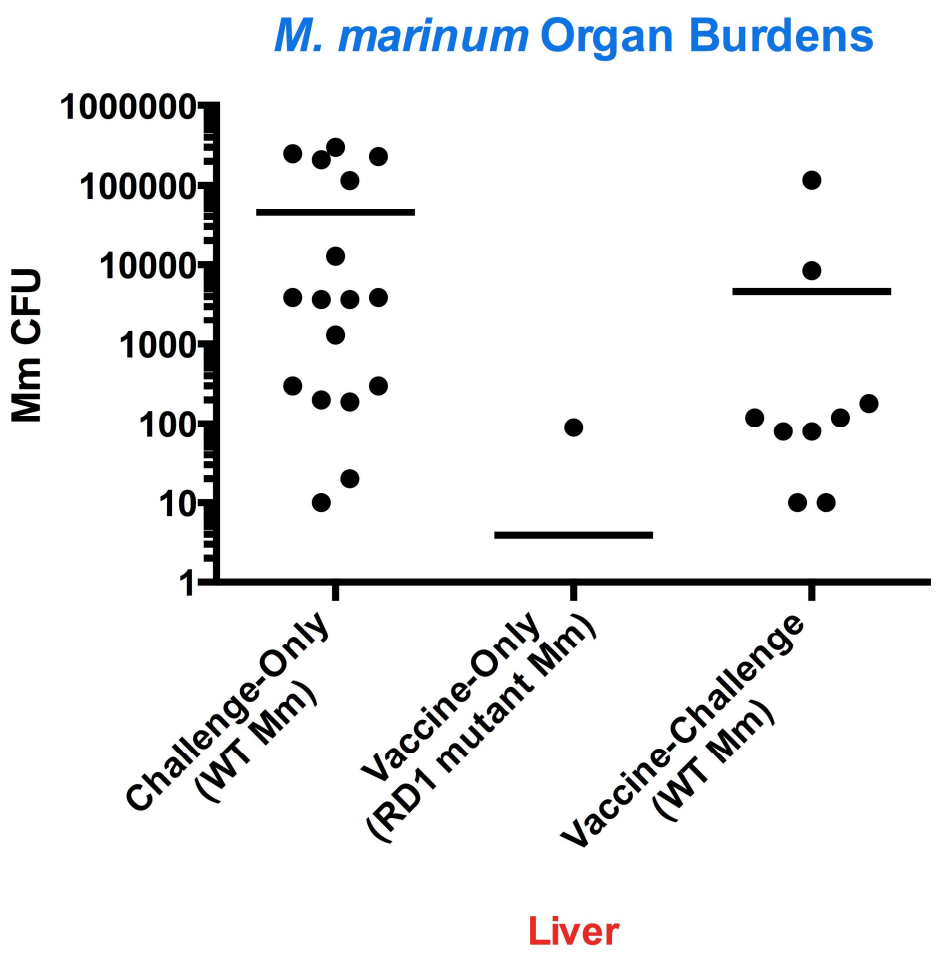


Figure 3.5: Protective effects of vaccination on liver burdens. Liver burdens in individual fish are comparing initially vaccinated and then challenged by the *rfp*-marked wild-type strain (DE4373) to burdens of the ΔRD1 mutant (DE4558) in fish that only received the vaccination to the wild-type burdens in fish which did no benefit from vaccination. Black bars represent the statistical average of each group. Dots represent individual burdens. Not shown are all "zeros." All culture-negative "zero" results correspond to <10 CFU per organ. This graphical representation of these data was produced by using the Prism 6 statistical analysis program.

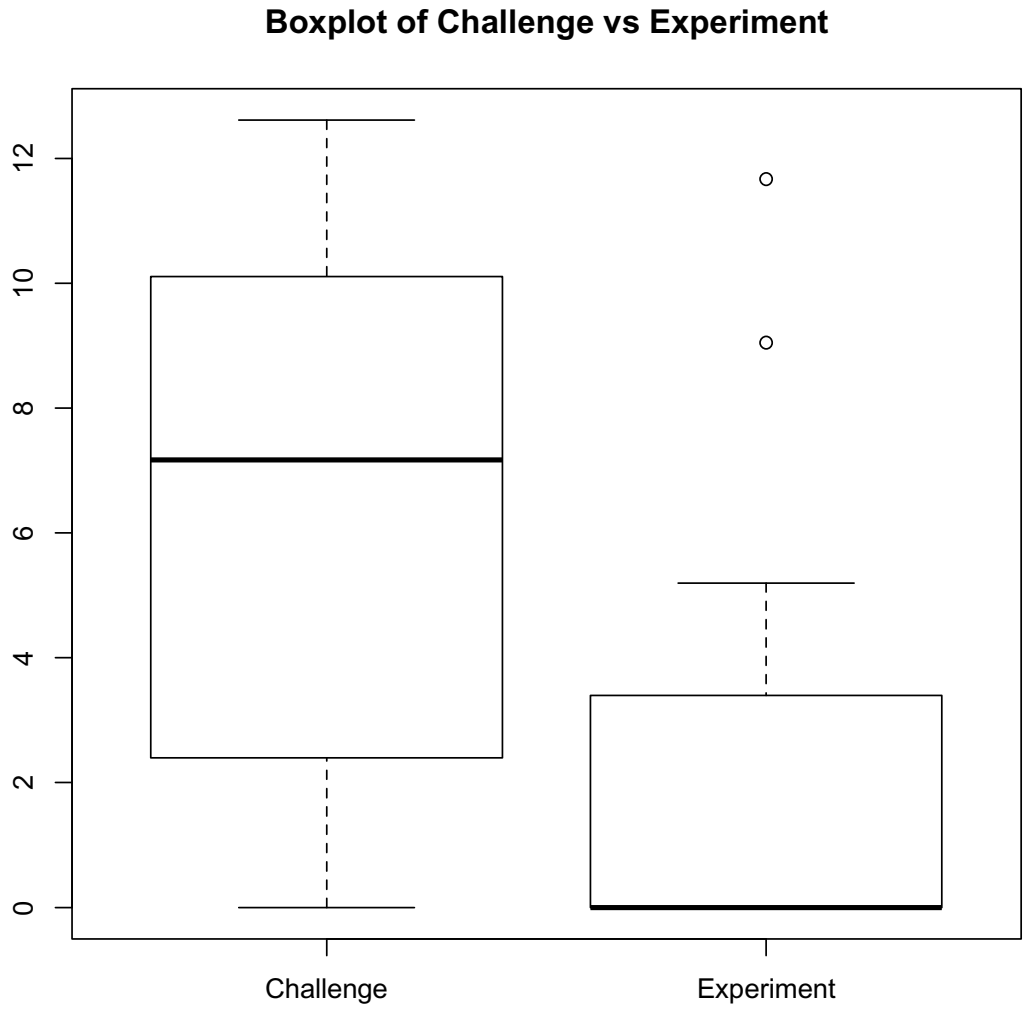


Figure 3.6: Boxplot representation of protective effects of vaccination on liver burdens. Liver burdens in individual fish are comparing initially vaccinated and then challenged by the *rfp*-marked wild-type strain (i.e., experiment) (DE4373) to the wild-type burdens in fish which did no benefit from vaccination (i.e., challenge). The black line represents the relative averages of the data. The boxes represent the limits of the 2nd and 3rd statistical quartiles. The error bars represent the full range of data. The two dots in the experimental group represent statistical outliers. The All culture-negative "zero" results correspond to <10 CFU per organ. Analysis performed by Avishek Mallick.

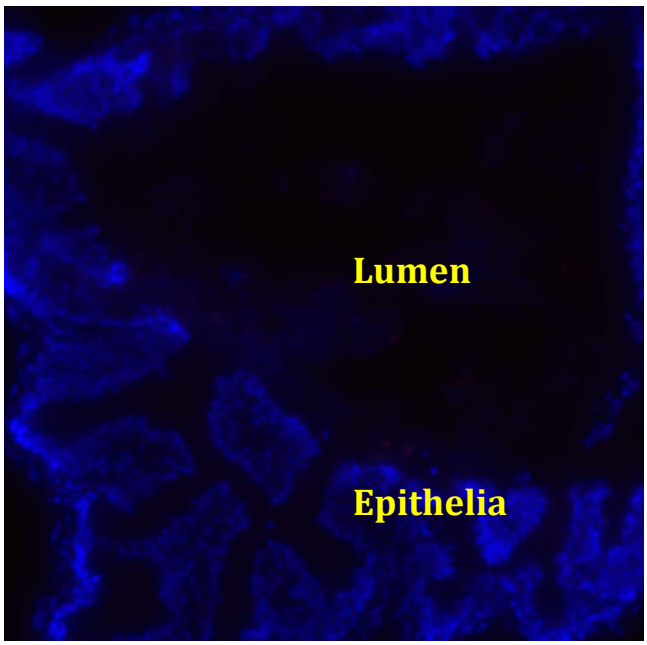


Figure 3.7: Uninfected medaka gut. A representative image of a frozen section of a dissected gut sample that had been treated with DAPI. As shown in Figure 3.2, the nuclei of the gut epithelia are stained blue. Image provided by Irene Salinas.

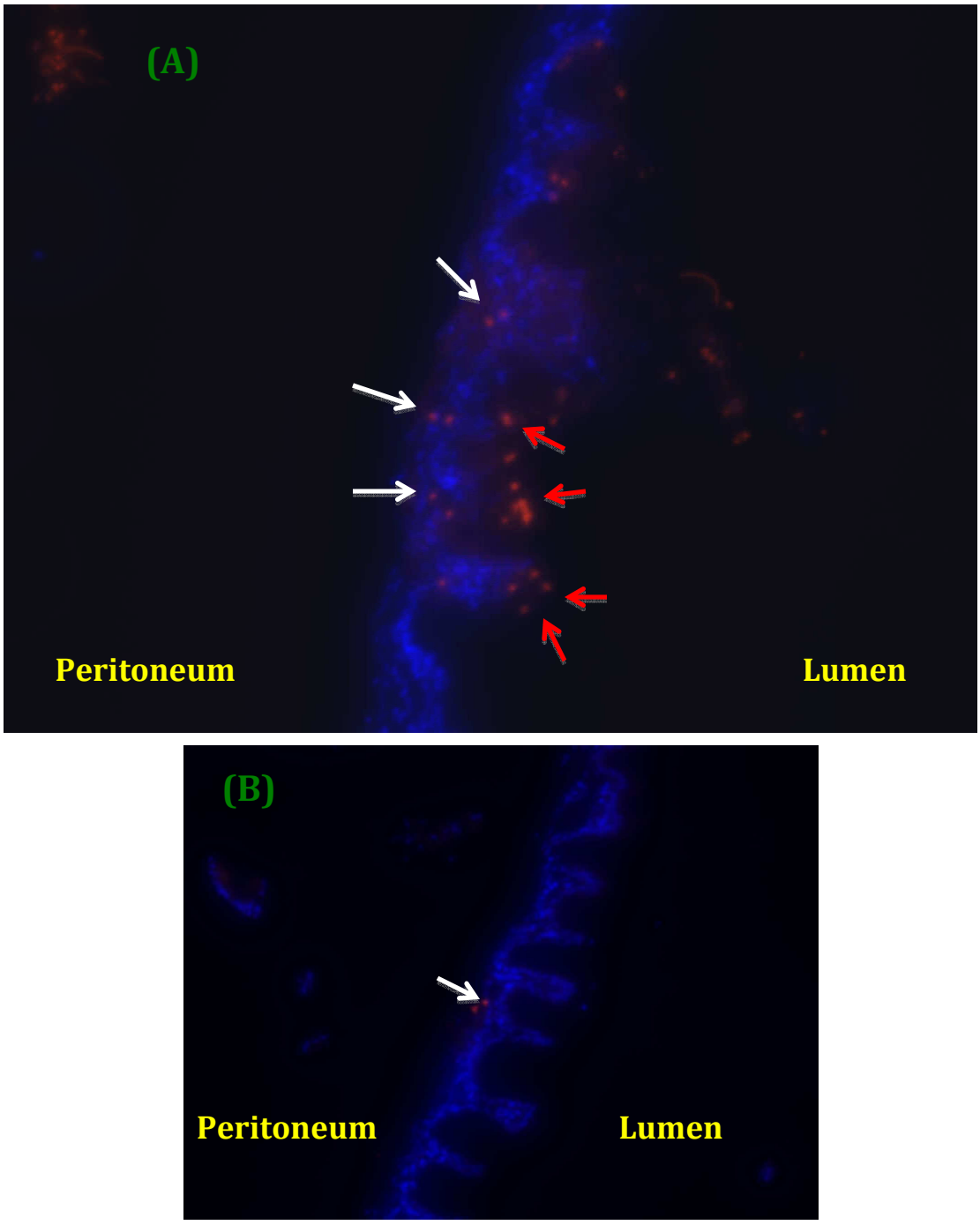


Figure 3.8: Infected gut tissues by virulent *rfp*-marked *M. marinum*. Represntative fluorescence images of antierior portion of medaka gut that was orally infected by the *rfp*-marked wild-type *M. marinum* strain (DE4373) three months prior. In (A), numerous foci of infections can be observed within gut tissue, but also, some red fluorescent aggregates appear to be on the surface of the epithelial layer. (B) shows two foci of infections embedded deeply in the gut tissue, possibly on the basal (peritoneal) surface of the gut. Red arrows represent luminal foci of infections, and white arrows represent foci of infections in underlying tissues. Images provided by Irene Salinas.

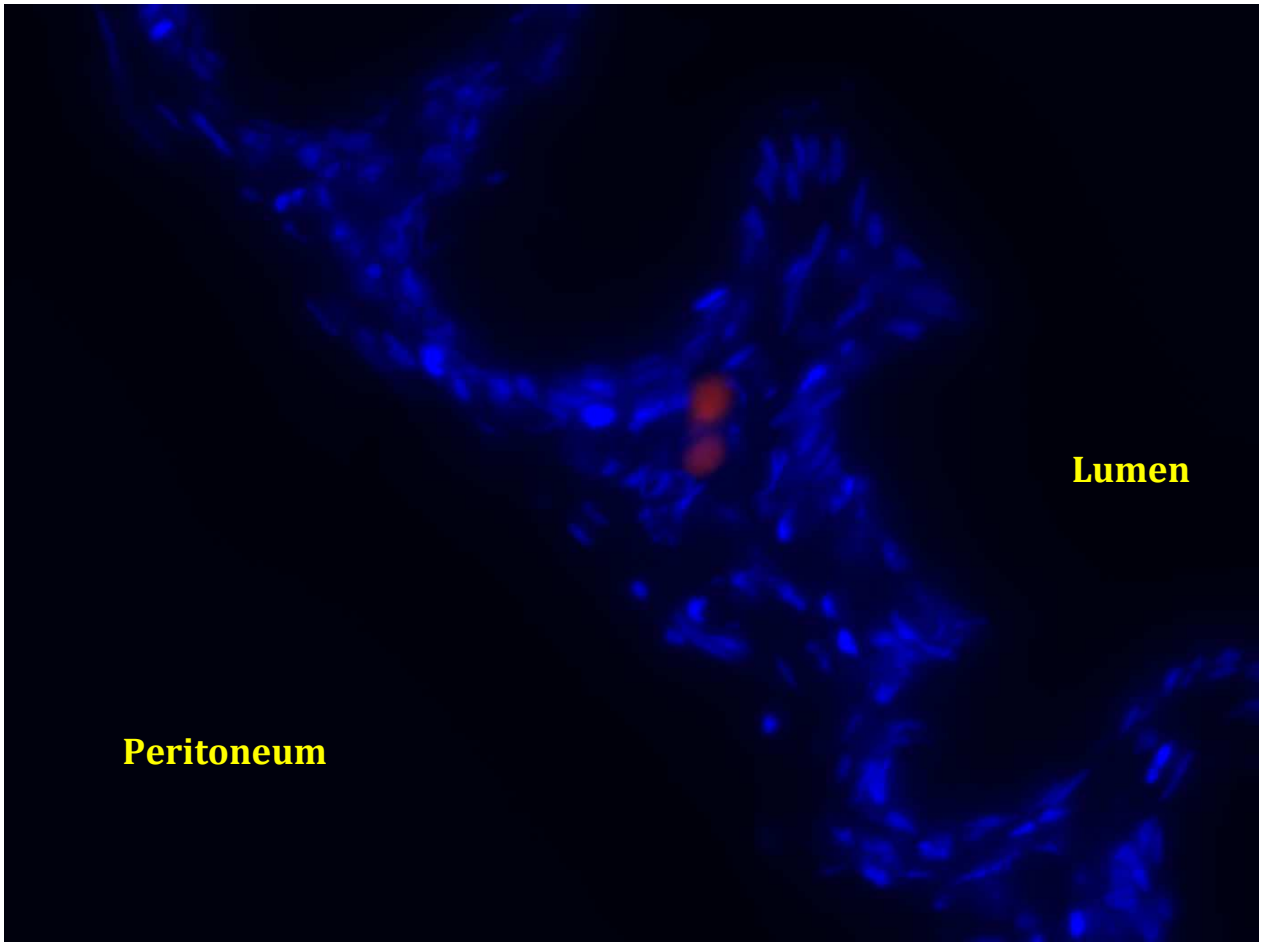


Figure 3.9: Foci of infections in the intestinal lamina propria. Dissected gut from fish orally infected by *rfp*-marked bacteria for three months. Image of magnified field of view revealing two prominent clusters of fluorescent bacteria embedded in gut tissue. Image provided by Irene Salinas

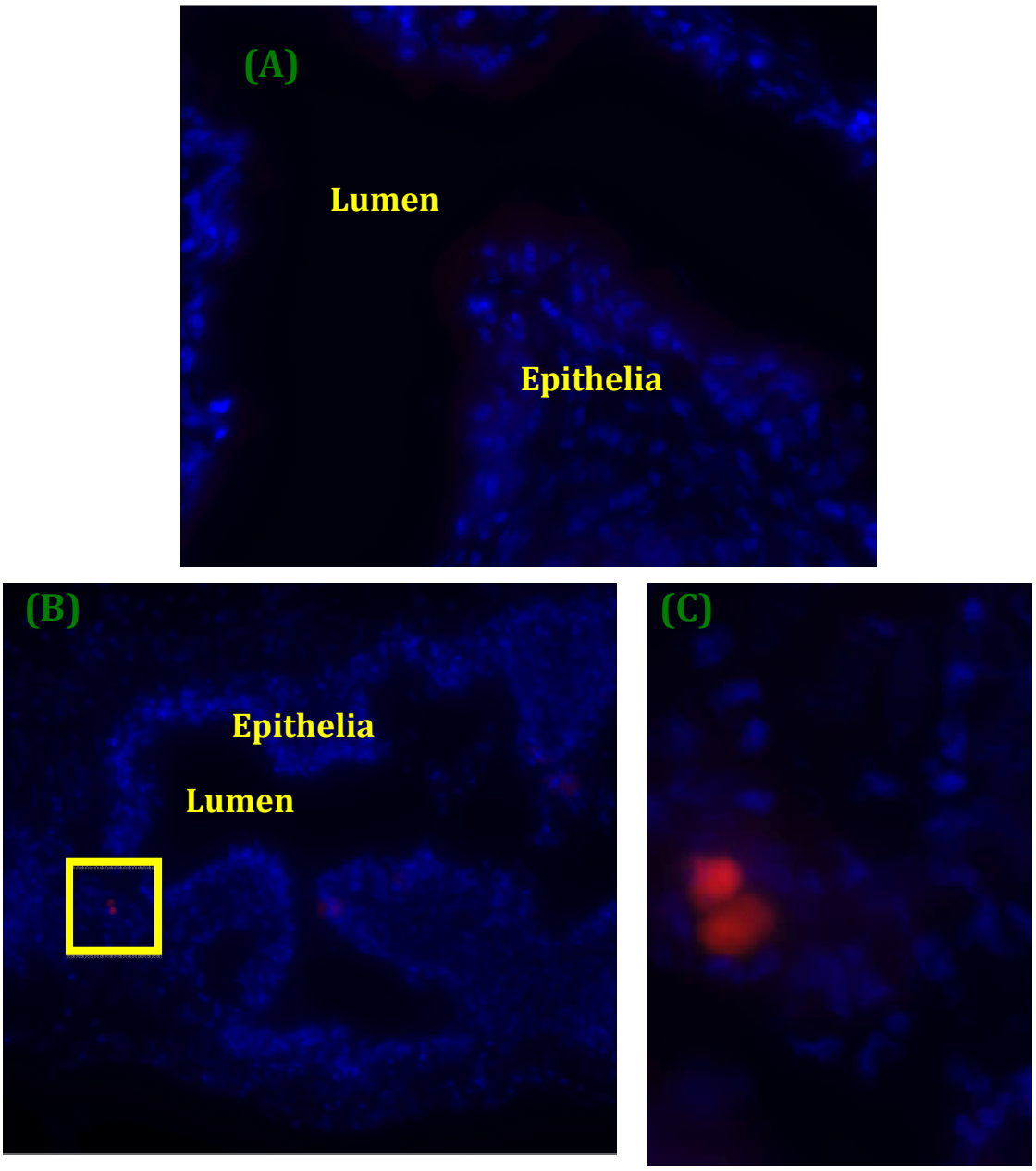


Figure 3.10: Vaccination greatly diminishes mycobacterial infections of the gut epithelia. Fish first orally exposed to the avirulent ΔRD1 mutant strain and then challenged to the *rfp*-marked virulent *M. marinum*. Two months post challenge, the gut was dissected from six fish and were frozen and then treated with DAPI and prepared for microscopy inspection. (A) is a representative image showing no red foci of infections. In all vaccinated gut sections examined from all fish studied, only one field of view revealed red foci of infections (B and C). Red arrows indicate other noticeable foci; however, these foci of infections cannot be identified as luminal or sub-epithelial foci. (B) represents one field of view with a foci in the epithelia and at a higher magnification (C), higher magnification image of the pair of fluorescent foci seen in (B). Images provided by Irene Salinas.

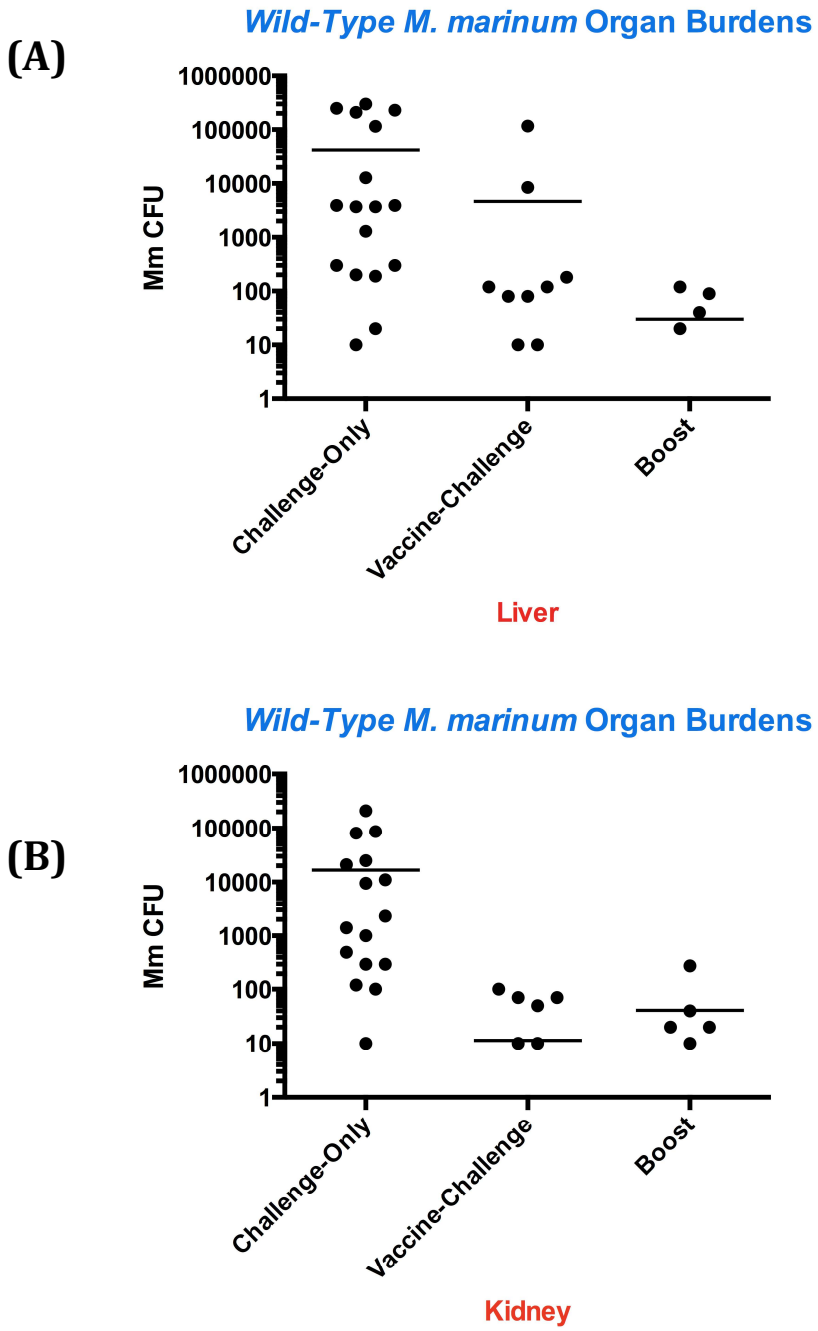
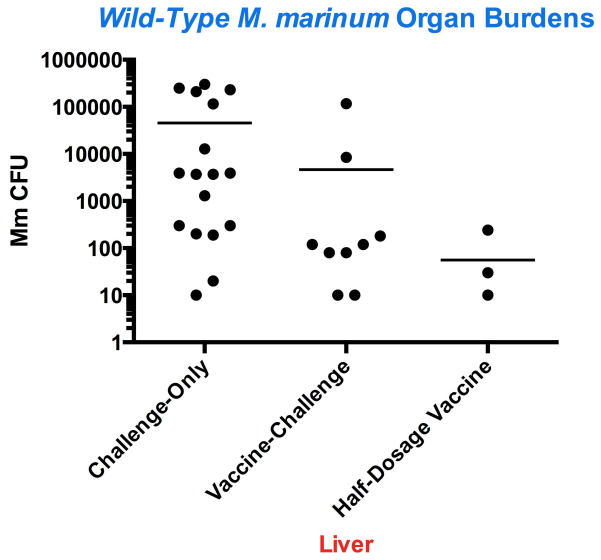


Figure 3.11: Boost regimen may provide additional protection against oral wild-type challenge. (A) compares liver burdens between challenge-only fish, vaccine-challenge fish, and boosted fish. (B) compares kidney burdens between these groups. While boosted fish appear to be protected, whether the boost confers additional protection remains unknown. Increasing the sample size of boosted fish may help offer additional insights into the protective quality of a boost. Not shown are all "zeros." All culture-negative "zero" results correspond to <10 CFU per organ. The challenge-only and vaccine-challenge data are also shown in Figures 3.3. and 3.4. This graphical representation of these data was produced by using the Prism 6 statistical analysis program.

(A)



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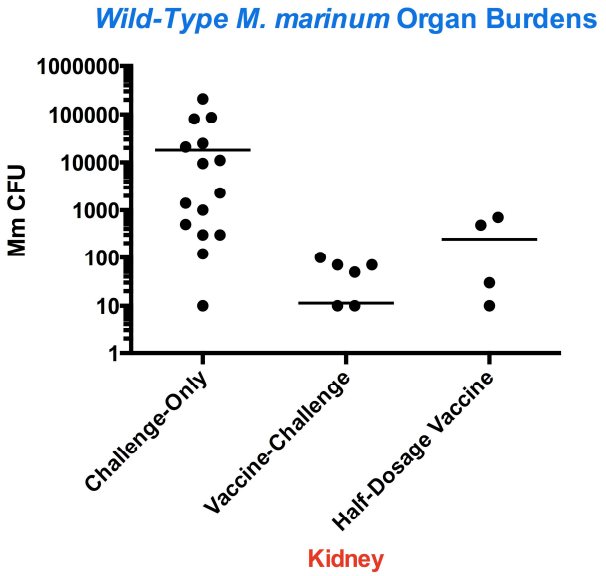
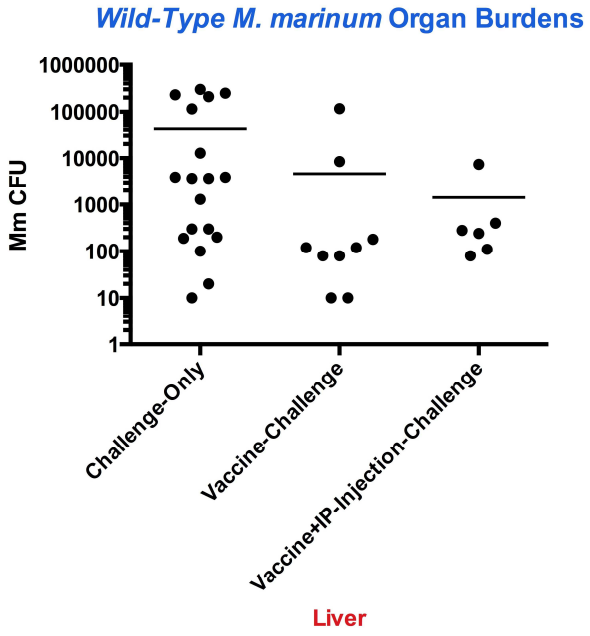


Figure 3.12: Half dosage of the ΔRD1 mutant confers protection against oral wild-type challenge. (A) compares liver burdens between challenge-only fish, vaccine-challenge fish, and half-dose vaccinated fish. (B) compares kidney burdens between these groups. While two meals of larvae carrying the ΔRD1 mutant appear to be protected, whether the half-dose vaccination confers adequate protection remains unknown. Increasing the sample size would be required to determine if the same level of protection is conferred as the "full dose" vaccine-challenge group. Not shown are all "zeros." All culture-negative "zero" results correspond to <10 CFU per organ. The challenge-only and vaccine-challenge data are also shown in Figures 3.3. and 3.4. This graphical representation of these data was produced by using the Prism 6 statistical analysis program.

(A)



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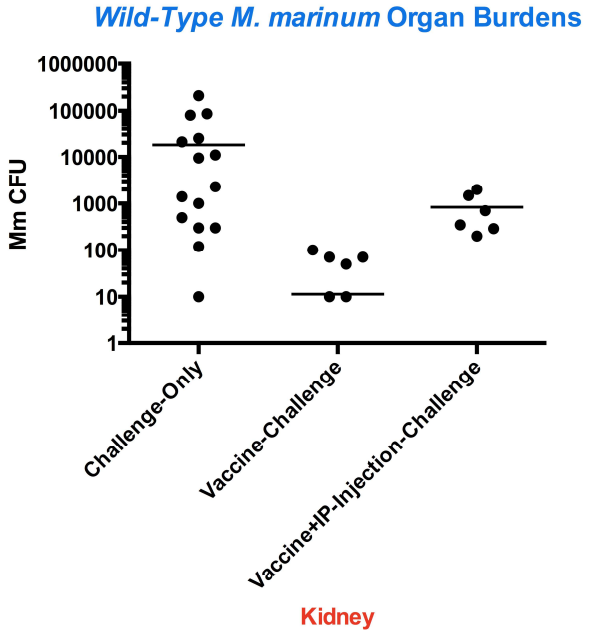

Figure 3.13: oral vaccination provides some protection against an IP-injected wild-type challenge. (A) compares liver burdens between challenge-only fish, vaccine-challenge fish, and IP-injection-challenge fish. (B) compares kidney burdens between these groups. While orally vaccinated fish appear to be protected against an IP-injected challenge, whether the oral vaccination confers systemic protection remains unknown. Increasing the sample size may help offer additional insights into the protective quality of a reduced vaccine strain inoculation. Not shown are all "zeros." All culture-negative "zero" results correspond to <10 CFU per organ. The challenge-only and vaccine-challenge data are also shown in Figures 3.3. and 3.4. This graphical representation of these data was produced by using the Prism 6 statistical analysis program.

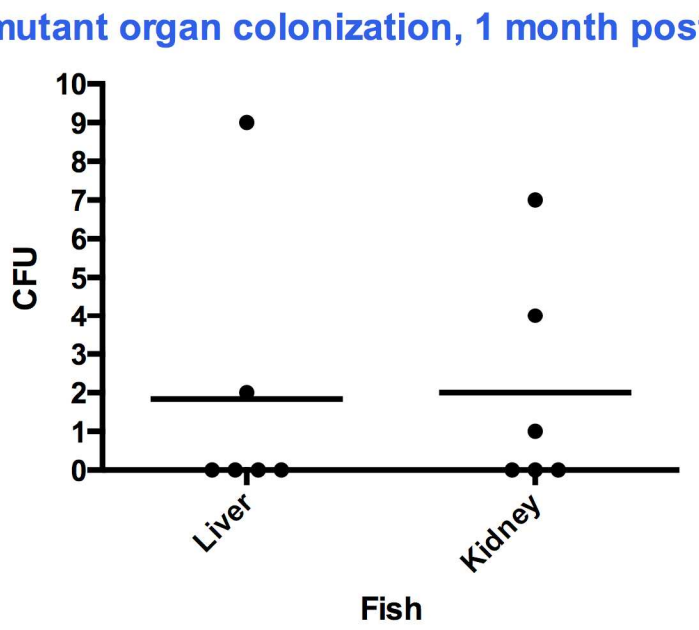

Figure 3.14: persistence of vaccine strain in organs one month after inoculation. Fish were exposed to the ΔRD1 mutant vaccine strain (DE4558) by ingestion of four meals of infected mosquito larvae. At four weeks, target organs were dissected, homogenized, and then plated for colony counts. Small, slow-growing CFU characteristic of this mutant strain were observed after approximately two weeks of incubation. Black bars represent the average burden, while dots represent individual burdens. Three of the six fish were culture positive for the ΔRD1 mutant. This graphical representation of these data was produced by using the Prism 6 statistical analysis program.

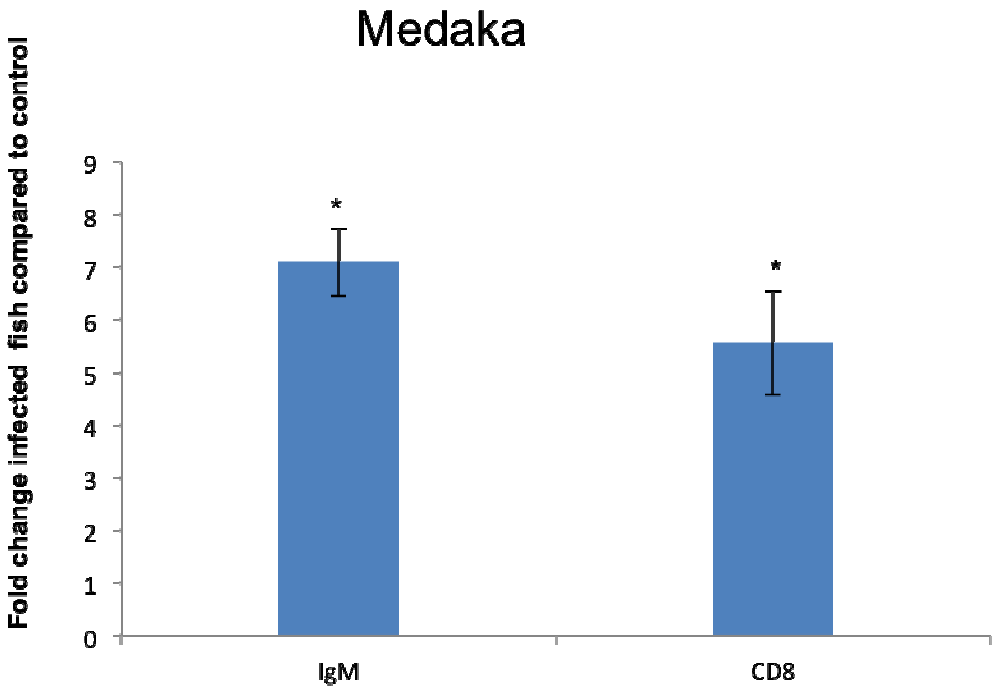

Figure 3.15: Exposure to ΔRD1 mutant strain (DE4558) leads to upregulation of IgM and CD8 gene expression. Fish were exposed to the ΔRD1 mutant vaccine strain (DE4558) by ingestion of four meals of infected mosquito larvae. Four weeks after exposure, gut samples were dissected out, placed in RNA easy, and frozen at -80° C, and the samples were sent to our collaborator Irene Salinas to determine if CD8 and IgM genes were upregulated in the gut compared to gut samples from unvaccinated, control fish. The histogram was provided by Irene Salinas.

Chapter 4

Discussion of Results

4.1. The role(s) of the gut mucosal interface as a portal for mycobacterial infection

Though *M. marinum* has great commercial impact and scientific appeal, until recently, little was known about the natural routes of infection by *M. marinum* in fish. Recent research suggests that a prominent route is oral, and in turn, there is an important interaction with the pathogen and the gastrointestinal tract (Mutoji, 2011). This suggests that an intimate interaction in the gut between host cells and invading bacterial cells leads to local and possibly systemic infection, resulting in the colonization of organs. Hence, the pathogen must not only evolve techniques to attach then invade the gut mucosal layer and evade killing by GALT immune cells but likely exploit these cells, like macrophages, to transport the bacteria to the viscera.

Indeed, gut-associated modes of infections by a few model systems have been previously characterized. For example, *Yersenia pseudotuberculosis* is a well-documented food-borne pathogen. *Y. pseudotuberculosis* is able to establish systemic infection by high-jacking aspects of the host's mucosal immune system. The pathogen targets and attaches to Peyer's patches then, induces the M cells to engulf these bacteria. Upon delivery to the associated macrophages in the GALT, the bacteria that are able to evade killing is then released into the interior and successfully mount infection. Moreover, some mycobacterial species (e.g., MAP species and *M. bovis*) are also able to infect ruminants via an oral route of infection (i.e., Johne's disease), presumably also exploiting the GALT system (Wilson et al., 2011). Since many

mycobacterial pathogens also reside in macrophages, infection of the phagocytes of the GALT system have been implicated for mycobacterial infections. The GALT-like systems are less well understood in fish systems, although some components have been documented (Flajnik, 2010). Therefore, It is plausible that the fish GALT is also exploited by *M. marinum*. Research has shown that fish actively shed *M. marinum*, and that mycobacteria are present in the posterior gut lumen in large quantities (Mutoji, 2011, Root, 2012, and my observations). The active shedding implicates shedding as a means for continued *M. marinum* presence in aquatic environments and thus, re-exposure of the pathogen to other susceptible hosts (Mutoji, 2011; Akleh et al., 2014). Possible exposure routes to *M. marinum* after shedding include direct exposure to other fish, ingestion of infected feces, and intermediate hosts that may ingest these bacteria (e.g., mosquito larvae), which in turn are preyed upon by susceptible fish (Ackleh et al., 2014). These additional considerations suggest that interaction of this pathogen with the gut epithelia, as well as mucosal immunity, may play a critical role in the infection of fish.

A major conclusion of my thesis is the documentation that *M. marinum* is able to cross the gut epithelia in a relatively short time-frame and then, travel to the underlying gut tissues. Fluorescence microscopy of frozen gut tissue samples prepared after oral exposure suggest that penetration of the host gut epithelia by *M. marinum* is relatively quick. For example, in one-week post infection samples, bacterial aggregates or foci of infection were found deep within the underlying tissues, like the submucosa. The aggregates could be nascent granulomas and would support the view by Volkman et al. that the innate immune system plays a role in initial granuloma formation. Further

studies into these early events may unveil novel virulence factors and elucidate the molecular mechanisms used by *M. marinum* to cross the epithelia, interact with host macrophages, and gain access to target tissues via transportation by the circulatory system. Also, characterizing short- and long-term immune responses (i.e., immunoglobin induction) could help identify protective roles of the host immune responses after exposure to mycobacteria, which remains an active area of mycobacterial research and vaccine design. *M. tuberculosis* invades the mucosal linings of the lungs to mount infection in humans; the bacterium interacts with the MALT system cells in the lungs' mucosa. Exploring vaccine treatments developed from stimulating mucosal immunity, which accounts for the majority of immune responses in humans, to provide long-term immunity against Mtb, may help reduce infection rates in humans by producing protective antibodies in the mucosal linings of the lungs.

4.2. *M. marinum* candidate vaccine strain virulence

The ΔRD1 interval on the genomes of many mycobacterial pathogens was initially defined by a deletion found in the BCG vaccine strain. The genes found in this interval, such as ESAT-6 and CFP-10, were since found to play critical roles in virulence, granuloma formation, and persistence. Researchers infected human macrophage cells with the ΔRD1 mutant *M. marinum* and wild-type *M. marinum* to compare bacterial loads. At ten days post-infection, researchers detected 1000-fold less ΔRD1 mutant *M. marinum* than wild-type *M. marinum* Bacterial loads in frog spleens were compared, and the ΔRD1 mutant was shown to be 10 times less virulent than the wild-type strain (Volkman et al., 2004). Furthermore, the RD1 locus was characterized to house genes responsible for mediating macrophage aggregation. Zebrafish infected with a

fluorescence-marked ΔRD1 mutant *M. marinum* strain formed significantly less aggregates in infected tissues compared to the wild-type strain. The researchers explain that aggregation of immune cells leads to increased infectivity due to the recruitment of susceptible phagocytic cells to the aggregates. The research discovered that granuloma formation enhances mycobacterial virulence. Additional experiments revealed that *M. marinum* and *M. tuberculosis* RD1 locus are interchangeable and complement each other (Volkman et al., 2004).

Consistent with other host infection models, my experiments show that the ΔRD1 mutant of *M. marinum* strain also confers greatly reduced virulence in medaka. Organ bacterial loads for the attenuated strain are significantly reduced when compared to the wild-type *M. marinum*; indeed, the mutant is often difficult to retrieve from fish organs; the ΔRD1 mutant is likely to be cleared by the host immune response. Furthermore, competition experiments between the wild-type and ΔRD1 mutant *M. marinum* strains shows that ΔRD1 mutant is 10-fold to 10,000-fold less virulent than the wild-type strain (Root, 2012).

4.3. Vaccination

As noted, BCG's protective qualities as a human vaccine against TB remain controversial, though BCG is the best available and most common anti-TB vaccine. Additionally, anti-*M. marinum* vaccines for fish have not yet been developed, and *M. marinum* infection remains a major cause of loss of food fish and has large economic impact to the commercial fishing and aquaculture industries. An effective anti-*M. marinum* vaccine for food fish would be expected to be a great benefit aquaculture. Moreover, modeling vaccines in the tractable *M. marinum* system should offer insights

for researchers to investigate improved anti-TB vaccine candidates, as well as vaccines for mycobacterial diseases in cattle.

The statistical analysis of my data presented here offers support for the conclusion that the ΔRD1 mutant strain provides protection against a wild-type *M. marinum* challenge. Vaccinated fish were protected against challenges by the wild-type *M. marinum*; moreover, wild-type bacterial loads were significantly different between vaccinated and unvaccinated fish by several orders of magnitude in essentially all cases. Though the boost group seemed equally protected against a wild-type challenge, the boost does not convincingly provide additional protection due to the small number of fish that were studied. However, a higher number of test hosts (i.e., significantly greater than n = 9) may help clarify if there might be a small additional protection provided with a boost regimen. In retrospect, the boost may be of little help to augment the immune system, as my studies and past studies have shown that the wild-type *M. marinum* continue to colonize the gut for at least 11 weeks post exposure (Mutoji, 2011). However, the timelines chosen for vaccinations, challenges, and booster dosages was picked somewhat arbitrarily and results may vary with alternate time points and regimens. Though, it remains unknown whether the ΔRD1 mutant is able to colonize the gut lumen for an extent amount of time after infection. This may be an important parameter for future studies, to gain insights into whether a boost would be beneficial.

Future experiments investigating the protective quality of the ΔRD1 mutant include: exposing vaccinated fish to higher acute dosage of wild-type bacteria during the challenge to use alternate end points and test if the vaccine strain also offers protection against acute disease and mortality. Challenging orally vaccinated fish via IP-

injection with wild-type *M. marium* would help clarify if the protective immune response is confined to the GI tract or not. It would also be interesting to investigate if fish immune systems are able to induce long-term protective and systemic immunity against wild-type *M. marinum* if primed with the ΔRD1 mutant via IP injections.

Another interesting avenue for future experiments would be to further attenuate the vaccine strain candidate by inactivating other virulence genes in the ΔRD1 mutant background. Past experiments by Mutoji and Ennis (2012) showed that over expression of *gfp* is toxic to *M. marinum* and significantly reduces virulence in medaka. Perhaps, vaccinating fish with a construct with the *gfp*-marked ΔRD1 mutant may offer even greater attenuation but still offer protection. Improved vaccine design also offers interesting potential for collaborations with other labs. For example, mutants in the immunogenic gene (*hip1*), in combination with the ΔRD1 deletion might offer improved protective immunity. The ΔRD1 mutant vaccine candidate or its multiple mutant derivatives could then be tested in the *in vivo* medaka system in a cost effective manner. Along these lines, it would be interesting to characterize if experimental infections by BCG in medaka also offers protective immunity to subsequent wild-type *M. marinum* challenge.

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ABSTRACT

Mycobacterium marinum is an established surrogate pathogen for *Mycobacterium tuberculosis* because of *M. marinum*'s strong conservation of thousands of orthologous genes, lower risk, lower financial burden to researchers, and similar pathology in fish. This pathogen causes TB-like chronic disease in a wide variety of fish species and can mount superficial infection of human tissues. As in human TB, the microbe grows within the host macrophages, can mount life-long chronic infections, and produces granulomatous lesions in target organs. One of the fish species known to manifest chronic "fish TB" is the small laboratory fish, Japanese medaka (*Oryzias latipes*). Recently, our lab documented the progression of the bacterium from the lumen of the gut to underlying tissues and to target organs to mount infection. Since the bacterium can be observed crossing the epithelia to mount infection, I tested to see if mucosal immunity against a wild-type challenge could be induced by initially priming the fish to a live, attenuated vaccine strain. This thesis demonstrates that inoculation by ingestion is an efficient mode by which medaka can become infected and vaccinated with *M. marinum*. Furthermore, my thesis shows that orally vaccinating fish with a live, attenuated strain indeed provides protection in the gut, liver, and kidney against a virulent, wild-type challenge.

BIOGRAPHICAL SKETCH

Martin Cheramie was born on a cold, snowy day, November 25, 1988, in Albi, France to David and Marianne Cheramie. He was the second of three boys. His family moved to Louisiana when he was an infant. Martin graduated high school in 2007 from Lafayette High School and college in 2011 from William Woods University. Martin enjoys the critical thinking, logic, and creativity involved with basic science research. When not doing research, Martin can most likely be found on a soccer field kicking a ball. Martin earned a Master's in Science from the University of Louisiana at Lafayette in 2014. He hopes to have a successful career in medicine.